

PREDICTING BREAST CANCER TREATMENT OUTCOME

Related Applications

This application claims benefit of priority from U.S. Provisional Patent Application 60/504,087, filed September 19, 2003, and is a continuation in part of U.S. Patent Application 10/727,100, filed December 2, 2003. Both applications are hereby incorporated by reference in their entireties as if fully set forth.

Field of the Invention

The invention relates to the identification and use of gene expression profiles, or patterns, with clinical relevance to the treatment of breast cancer using tamoxifen (nolvadex) and other “antiestrogen” agents against breast cancer, including other “selective estrogen receptor modulators” (“SERM”s), “selective estrogen receptor downregulators” (“SERD”s), and aromatase inhibitors (“AI”s). In particular, the invention provides the identities of gene sequences the expression of which are correlated with patient survival and breast cancer recurrence in women treated with tamoxifen or other “antiestrogen” agents against breast cancer. The gene expression profiles, whether embodied in nucleic acid expression, protein expression, or other expression formats, may be used to select subjects afflicted with breast cancer who will likely respond positively to treatment with tamoxifen or another “antiestrogen” agent against breast cancer as well as those who will likely be non-responsive and thus candidates for other treatments. The invention also provides the identities of three sets of sequences from three genes with expression patterns that are strongly predictive of responsiveness to tamoxifen and other “antiestrogen” agents against breast cancer.

Background of the Invention

Breast cancer is by far the most common cancer among women. Each year, more than 180,000 and 1 million women in the U.S. and worldwide, respectively, are diagnosed with breast cancer. Breast cancer is the leading cause of death for women between ages 50-55, and is the most

common non-preventable malignancy in women in the Western Hemisphere. An estimated 2,167,000 women in the United States are currently living with the disease (National Cancer Institute, Surveillance Epidemiology and End Results (NCI SEER) program, *Cancer Statistics Review (CSR)*, www-seer.ims.nci.nih.gov/Publications/CSR1973 (1998)). Based on cancer rates from 1995 through 1997, a report from the National Cancer Institute (NCI) estimates that about 1 in 8 women in the United States (approximately 12.8 percent) will develop breast cancer during her lifetime (NCI's Surveillance, Epidemiology, and End Results Program (SEER) publication *SEER Cancer Statistics Review 1973-1997*). Breast cancer is the second most common form of cancer, after skin cancer, among women in the United States. An estimated 250,100 new cases of breast cancer are expected to be diagnosed in the United States in 2001. Of these, 192,200 new cases of more advanced (invasive) breast cancer are expected to occur among women (an increase of 5% over last year), 46,400 new cases of early stage (*in situ*) breast cancer are expected to occur among women (up 9% from last year), and about 1,500 new cases of breast cancer are expected to be diagnosed in men (Cancer Facts & Figures 2001 American Cancer Society). An estimated 40,600 deaths (40,300 women, 400 men) from breast cancer are expected in 2001. Breast cancer ranks second only to lung cancer among causes of cancer deaths in women. Nearly 86% of women who are diagnosed with breast cancer are likely to still be alive five years later, though 24% of them will die of breast cancer after 10 years, and nearly half (47%) will die of breast cancer after 20 years.

Every woman is at risk for breast cancer. Over 70 percent of breast cancers occur in women who have no identifiable risk factors other than age (U.S. General Accounting Office. Breast Cancer, 1971-1991: Prevention, Treatment and Research. GAO/PEMD-92-12; 1991). Only 5 to 10% of breast cancers are linked to a family history of breast cancer (Henderson IC, Breast Cancer. In: Murphy GP, Lawrence WL, Lenhard RE (eds). *Clinical Oncology*. Atlanta, GA: American Cancer Society; 1995:198-219).

Each breast has 15 to 20 sections called lobes. Within each lobe are many smaller lobules. Lobules end in dozens of tiny bulbs that can produce milk. The lobes, lobules, and bulbs are all linked by thin tubes called ducts. These ducts lead to the nipple in the center of a dark area of skin called the areola. Fat surrounds the lobules and ducts. There are no muscles in the breast, but muscles lie under each breast and cover the ribs. Each breast also contains blood vessels and lymph

vessels. The lymph vessels carry colorless fluid called lymph, and lead to the lymph nodes. Clusters of lymph nodes are found near the breast in the axilla (under the arm), above the collarbone, and in the chest.

Breast tumors can be either benign or malignant. Benign tumors are not cancerous, they do not spread to other parts of the body, and are not a threat to life. They can usually be removed, and in most cases, do not come back. Malignant tumors are cancerous, and can invade and damage nearby tissues and organs. Malignant tumor cells may metastasize, entering the bloodstream or lymphatic system. When breast cancer cells metastasize outside the breast, they are often found in the lymph nodes under the arm (axillary lymph nodes). If the cancer has reached these nodes, it means that cancer cells may have spread to other lymph nodes or other organs, such as bones, liver, or lungs.

Major and intensive research has been focused on early detection, treatment and prevention. This has included an emphasis on determining the presence of precancerous or cancerous ductal epithelial cells. These cells are analyzed, for example, for cell morphology, for protein markers, for nucleic acid markers, for chromosomal abnormalities, for biochemical markers, and for other characteristic changes that would signal the presence of cancerous or precancerous cells. This has led to various molecular alterations that have been reported in breast cancer, few of which have been well characterized in human clinical breast specimens. Molecular alterations include presence/absence of estrogen and progesterone steroid receptors, HER-2 expression/amplification (Mark HF, et al. HER-2/neu gene amplification in stages I-IV breast cancer detected by fluorescent in situ hybridization. *Genet Med*; 1(3):98-103 1999), Ki-67 (an antigen that is present in all stages of the cell cycle except G0 and used as a marker for tumor cell proliferation, and prognostic markers (including oncogenes, tumor suppressor genes, and angiogenesis markers) like p53, p27, Cathepsin D, pS2, multi-drug resistance (MDR) gene, and CD31.

Tamoxifen is the antiestrogen agent most frequently prescribed in women with both early stage and metastatic hormone receptor-positive breast cancer (for reviews, see Clarke, R. et al. "Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling." *Oncogene* 22, 7316-39 (2003) and Jordan, C. "Historical perspective on hormonal therapy of advanced breast Cancer." *Clin. Ther.* 24 Suppl A, A3-16 (2002)). In the adjuvant setting, tamoxifen therapy results

in a 40-50% reduction in the annual risk of recurrence, leading to a 5.6% improvement in 10 year survival in lymph node negative patients, and a corresponding 10.9% improvement in node-positive patients (Group, E.B.C.T.C. Tamoxifen for early breast cancer. Cochrane Database Syst Rev, CD000486 (2001)). Tamoxifen is thought to act primarily as a competitive inhibitor of estrogen binding to estrogen receptor (ER). The absolute levels of ER expression, as well as that of the progesterone receptor (PR, an indicator of a functional ER pathway), are currently the best predictors of tamoxifen response in the clinical setting (Group, (2001) and Bardou, V.J. et al. "Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases." J Clin Oncol 21, 1973-9 (2003)).

However, 25% of ER+/PR+ tumors, 66% of ER+/PR- cases and 55% of ER-/PR+ cases fail to respond, or develop early resistance to tamoxifen, through mechanisms that remain largely unclear (see Clarke et al.; Nicholson, R.I. et al. "The biology of antihormone failure in breast cancer." Breast Cancer Res Treat 80 Suppl 1, S29-34; discussion S35 (2003) and Osborne, C.K. et al. "Growth factor receptor cross-talk with estrogen receptor as a mechanism for tamoxifen resistance in breast cancer." Breast 12, 362-7 (2003)). Currently, no reliable means exist to allow the identification of these non-responders. In these patients, the use of alternative hormonal therapies, such as the aromatase inhibitors letrozole and anastrozole (Ellis, M.J. et al. "Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptorpositive primary breast cancer: evidence from a phase III randomized trial." J Clin Oncol 19, 3808-16 (2001); Buzdar, A.U. "Anastrozole: a new addition to the armamentarium against advanced breast cancer." Am J Clin Oncol 21, 161-6 (1998); and Goss, P.E. et al. "A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer." N Engl J Med 349, 1793-802 (2003)); chemotherapeutic agents, or inhibitors of other signaling pathways, such as trastuzumab and gefitinib might offer the possibility of improving clinical outcome. Therefore, the ability to accurately predict tamoxifen treatment outcome should significantly advance the management of early stage breast cancer by identifying patients who are unlikely to benefit from TAM so that additional or alternative therapies may be sought.

Citation of documents herein is not intended as an admission that any is pertinent prior art. All statements as to the date or representation as to the contents of documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of the documents.

5 Summary of the Invention

 The present invention relates to the identification and use of gene expression patterns (or profiles or “signatures”) and the expression levels of individual gene sequences which are clinically relevant to breast cancer. In particular, the identities of genes that are correlated with patient survival and breast cancer recurrence (e.g. metastasis of the breast cancer) are provided. The gene
10 expression profiles, whether embodied in nucleic acid expression, protein expression, or other expression formats, may be used to predict survival of subjects afflicted with breast cancer and the likelihood of breast cancer recurrence, including cancer metastasis.

 The invention thus provides for the identification and use of gene expression patterns (or profiles or “signatures”) and the expression levels of individual gene sequences which correlate
15 with (and thus are able to discriminate between) patients with good or poor survival outcomes. In one embodiment, the invention provides patterns that are able to distinguish patients with estrogen receptor (α isoform) positive (ER+) breast tumors into those with that are responsive, or likely to be responsive, to treatment with tamoxifen (TAM) or another “antiestrogen” agent against breast cancer (such as a “selective estrogen receptor modulator” (“SERM”), “selective estrogen receptor
20 downregulator” (“SERD”), or aromatase inhibitor (“AI”)) and those that are non-responsive, or likely to be non-responsive, to such treatment. In an alternative embodiment, the invention may be applied to patients with breast tumors that do not display detectable levels of ER expression (so called “ER-“ subjects) but where the patient will nonetheless benefit from application of the invention due to the presence of some low level ER expression. Responsiveness may be viewed in
25 terms of better survival outcomes over time. These patterns are thus able to distinguish patients with ER+ breast tumors into at least two subtypes.

 In a first aspect, the present invention provides a non-subjective means for the identification of patients with breast cancer (ER+ or ER-) as likely to have a good or poor survival outcome

following treatment with TAM or another “antiestrogen” agent against breast cancer by assaying for the expression patterns disclosed herein. Thus where subjective interpretation may have been previously used to determine the prognosis and/or treatment of breast cancer patients, the present invention provides objective gene expression patterns, which may used alone or in combination with subjective criteria to provide a more accurate assessment of ER+ or ER- breast cancer patient outcomes or expected outcomes, including survival and the recurrence of cancer, following treatment with TAM or another “antiestrogen” agent against breast cancer. The expression patterns of the invention thus provide a means to determine ER+ or ER- breast cancer prognosis. Furthermore, the expression patterns can also be used as a means to assay small, node negative tumors that are not readily assayed by other means.

The gene expression patterns comprise one or more than one gene capable of discriminating between breast cancer outcomes with significant accuracy. The gene sequence(s) are identified as correlated with ER+ breast cancer outcomes such that the levels of their expression are relevant to a determination of the preferred treatment protocols for a patient, whether ER+ or ER-. Thus in one embodiment, the invention provides a method to determine the outcome of a subject afflicted with breast cancer by assaying a cell containing sample from said subject for expression of one or more than one gene disclosed herein as correlated with breast cancer outcomes following treatment with TAM or another “antiestrogen” agent against breast cancer.

The ability to correlate gene expression with breast cancer outcome and responsiveness to TAM is particularly advantageous in light of the possibility that up to 40% of ER+ subjects that undergo TAM treatment are non-responders. Therefore, the ability to identify, with confidence, these non-responders at an early time point permits the consideration and/or application of alternative therapies (such as a different “antiestrogen” agent against breast cancer or other anti-breast cancer treatments) to the non-responders. Stated differently, the ability to identify TAM non-responder subjects permits medical personnel to consider and/or utilize alternative therapies for the treatment of the subjects before time is spent on ineffective TAM therapy. Time spent on an ineffective therapy often permits further cancer growth, and the likelihood of success with alternative therapies diminishes over time given such growth. Therefore, the invention also

provides methods to improve the survival outcome of non-responders by use of the methods disclosed herein to identify non-responders for treatment with alternative therapies.

Gene expression patterns of the invention are identified as described below. Generally, a large sampling of the gene expression profile of a sample is obtained through quantifying the expression levels of mRNA corresponding to many genes. This profile is then analyzed to identify genes, the expression of which are positively, or negatively, correlated, with ER+ breast cancer outcome upon treatment with TAM or another “antiestrogen” agent against breast cancer. An expression profile of a subset of human genes may then be identified by the methods of the present invention as correlated with a particular outcome. The use of multiple samples increases the confidence which a gene may be believed to be correlated with a particular survival outcome. Without sufficient confidence, it remains unpredictable whether expression of a particular gene is actually correlated with an outcome and also unpredictable whether expression of a particular gene may be successfully used to identify the outcome for a breast cancer patient. While the invention may be practiced based on the identities of the gene sequences disclosed herein or the actual sequences used independent of identification, the invention may also be practiced with any other sequences the expression of which is correlated with the expression of sequences disclosed herein. Such additional sequences may be identified by any means known in the art, including the methods disclosed herein.

A profile of genes that are highly correlated with one outcome relative to another may be used to assay an sample from a subject afflicted with breast cancer to predict the likely responsiveness (or lack thereof) to TAM or another “antiestrogen” agent against breast cancer in the subject from whom the sample was obtained. Such an assay may be used as part of a method to determine the therapeutic treatment for said subject based upon the breast cancer outcome identified.

As discussed below, the correlated genes may be used singly with significant accuracy or in combination to increase the ability to accurately correlating a molecular expression phenotype with a breast cancer outcome. This correlation is a way to molecularly provide for the determination of survival outcomes as disclosed herein. Additional uses of the correlated gene(s) are in the

classification of cells and tissues; determination of diagnosis and/or prognosis; and determination and/or alteration of therapy.

The ability to discriminate is conferred by the identification of expression of the individual genes as relevant and not by the form of the assay used to determine the actual level of expression.

5 An assay may utilize any identifying feature of an identified individual gene as disclosed herein as long as the assay reflects, quantitatively or qualitatively, expression of the gene in the “transcriptome” (the transcribed fraction of genes in a genome) or the “proteome” (the translated fraction of expressed genes in a genome). Additional assays include those based on the detection of polypeptide fragments of the relevant member or members of the proteome. Identifying features
10 include, but are not limited to, unique nucleic acid sequences used to encode (DNA), or express (RNA), said gene or epitopes specific to, or activities of, a protein encoded by said gene. All that is required are the gene sequence(s) necessary to discriminate between breast cancer outcomes and an appropriate cell containing sample for use in an expression assay.

In another embodiment, the invention provides for the identification of the gene expression
15 patterns by analyzing global, or near global, gene expression from single cells or homogenous cell populations which have been dissected away from, or otherwise isolated or purified from, contaminating cells beyond that possible by a simple biopsy. Because the expression of numerous genes fluctuate between cells from different patients as well as between cells from the same patient sample, multiple data from expression of individual genes and gene expression patterns are used as
20 reference data to generate models which in turn permit the identification of individual gene(s), the expression of which are most highly correlated with particular breast cancer outcomes.

In additional embodiments, the invention provides physical and methodological means for detecting the expression of gene(s) identified by the models generated by individual expression patterns. These means may be directed to assaying one or more aspects of the DNA template(s)
25 underlying the expression of the gene(s), of the RNA used as an intermediate to express the gene(s), or of the proteinaceous product expressed by the gene(s).

In further embodiments, the gene(s) identified by a model as capable of discriminating between breast cancer outcomes may be used to identify the cellular state of an unknown sample of cell(s) from the breast. Preferably, the sample is isolated via non-invasive means. The expression

of said gene(s) in said unknown sample may be determined and compared to the expression of said gene(s) in reference data of gene expression patterns correlated with breast cancer outcomes.

Optionally, the comparison to reference samples may be by comparison to the model(s) constructed based on the reference samples.

5 One advantage provided by the present invention is that contaminating, non-breast cells (such as infiltrating lymphocytes or other immune system cells) are not present to possibly affect the genes identified or the subsequent analysis of gene expression to identify the survival outcomes of patients with breast cancer. Such contamination is present where a biopsy is used to generate gene expression profiles. However, and as noted herein, the invention includes the identity of genes
10 that may be used with significant accuracy even in the presence of contaminating cells.

 In a second aspect, the invention provides a non-subjective means based on the expression of three genes, or combinations thereof, for the identification of patients with breast cancer as likely to have a good or poor survival outcome following treatment with TAM or another “antiestrogen” agent against breast cancer. These three genes are members of the expression patterns disclosed
15 herein which have been found to be strongly predictive of clinical outcome following TAM treatment of ER+ breast cancer.

 The present invention thus provides gene sequences identified as differentially expressed in ER+ breast cancer in correlation to TAM responsiveness. The sequences of two of the genes display increased expression in ER+ breast cells that respond to TAM treatment (and thus lack of
20 increased expression in nonresponsive cases). The sequences of the third gene display decreased expression in ER+ breast cells that respond to TAM treatment (and thus lack of decreased expression in nonresponsive cases).

 The first set of sequences found to be more highly expressed in TAM responsive, ER+ breast cells are those of interleukin 17 receptor B (IL17RB), which has been mapped to human
25 chromosome 3 at 3p21.1. IL17RB is also referred to as interleukin 17B receptor (IL17BR) and sequences corresponding to it, and thus may be used in the practice of the instant invention, are identified by UniGene Cluster Hs.5470.

 The second set of sequences found to be more highly expressed in TAM responsive, ER+ breast cells are those of the calcium channel, voltage-dependent, L type, alpha 1D subunit

(CACNA1D), which has been mapped to human chromosome 3 at 3p14.3. Sequences corresponding to CACNA1D, and thus may be used in the practice of the instant invention, are identified by UniGene Cluster Hs.399966.

The set of sequences found to be expressed at lower levels in TAM responsive, ER+ breast cells are those of homeobox B13 (HOXB13), which has been mapped to human chromosome 17 at 17q21.2. Sequences corresponding to HOXB13, and thus may be used in the practice of the instant invention, are identified by UniGene Cluster Hs.66731.

While the invention may be practiced based on the identities of these three gene sequences or the actual sequences used independent of the assigned identity, the invention may also be practiced with any other sequence the expression of which is correlated with the expression of these disclosed sequences. Such additional sequences may be identified by any means known in the art, including the methods disclosed herein.

The identified sequences may thus be used in methods of determining the responsiveness, or non-responsiveness, of a subject's ER+ or ER- breast cancer to TAM treatment, or treatment with another "antiestrogen" agent against breast cancer, via analysis of breast cells in a tissue or cell containing sample from a subject. As non-limiting examples, the lack of increased expression of IL17BR and CACNA1D sequences and/or the lack of decreased expression of HOXB13 sequences may be used as an indicator of nonresponsive cases. The present invention provides a non-empirical means for determining responsiveness to TAM or another SERM in ER+ or ER- patients. This provides advantages over the use of a "wait and see" approach following treatment with TAM or other "antiestrogen" agent against breast cancer. The expression levels of these sequences may also be used as a means to assay small, node negative tumors that are not readily assessed by conventional means.

The expression levels of the identified sequences may be used alone or in combination with other sequences capable of determining responsiveness to treatment with TAM or another "antiestrogen" agent against breast cancer. Preferably, the sequences of the invention are used alone or in combination with each other, such as in the format of a ratio of expression levels that can have improved predictive power over analysis based on expression of sequences corresponding to individual genes. The invention provides for ratios of the expression level of a sequence that is

underexpressed to the expression level of a sequence that is overexpressed as an indicator of responsiveness or non-responsiveness.

The present invention provides means for correlating a molecular expression phenotype with a physiological response in a subject with ER+ or ER- breast cancer. This correlation provides a way to molecularly diagnose and/or determine treatment for a breast cancer afflicted subject. Additional uses of the sequences are in the classification of cells and tissues; and determination of diagnosis and/or prognosis. Use of the sequences to identify cells of a sample as responsive, or not, to treatment with TAM or other “antiestrogen” agent against breast cancer may be used to determine the choice, or alteration, of therapy used to treat such cells in the subject, as well as the subject itself, from which the sample originated.

Such methods of the invention may be used to assist the determination of providing tamoxifen or another “antiestrogen” agent against breast cancer as a chemopreventive or chemoprotective agent to a subject at high risk for development of breast cancer. These methods of the invention are an advance over the studies of Fabian et al. (J Natl Cancer Inst. 92(15):1217-27, 2000), which proposed a combination of cytomorphology and the Gail risk model to identify high risk patients. The methods may be used in combination with assessments of relative risk of breast cancer such as that discussed by Tan-Chiu et al. (J Natl Cancer Inst. 95(4):302-307, 2003). Non-limiting examples include assaying of minimally invasive sampling, such as random (periareolar) fine needle aspirates or ductal lavage samples (such as that described by Fabian et al. and optionally in combination with or as an addition to a mammogram positive for benign or malignant breast cancer), of breast cells for the expression levels of gene sequences as disclosed herein to assist in the determination of administering therapy with an “antiestrogen” agent against breast cancer, such as that which may occur in cases of high risk subjects (like those described by Tan-Chiu et al.). The assays would thus lead to the identification of subjects for who the application of an “antiestrogen” agent against breast cancer would likely be beneficial as a chemopreventive or chemoprotective agent. It is contemplated that such application as enabled by the instant invention could lead to beneficial effects such as those seen with the administration of tamoxifen (see for example, Wickerham D.L., *Breast Cancer Res. and Treatment* 75 Suppl 1:S7-12, Discussion S33-5, 2000). Other applications of the invention include assaying of advanced breast cancer, including metastatic

cancer, to determine the responsiveness, or non-responsiveness, thereof to treatment with an “antiestrogen” agent against breast cancer.

An assay of the invention may utilize a means related to the expression level of the sequences disclosed herein as long as the assay reflects, quantitatively or qualitatively, expression of the sequence. Preferably, however, a quantitative assay means is preferred. The ability to determine responsiveness to TAM or other “antiestrogen” agent against breast cancer and thus outcome of treatment therewith is provided by the recognition of the relevancy of the level of expression of the identified sequences and not by the form of the assay used to determine the actual level of expression. Identifying features of the sequences include, but are not limited to, unique nucleic acid sequences used to encode (DNA), or express (RNA), the disclosed sequences or epitopes specific to, or activities of, proteins encoded by the sequences. Alternative means include detection of nucleic acid amplification as indicative of increased expression levels and nucleic acid inactivation, deletion, or methylation, as indicative of decreased expression levels. Stated differently, the invention may be practiced by assaying one or more aspect of the DNA template(s) underlying the expression of the disclosed sequence(s), of the RNA used as an intermediate to express the sequence(s), or of the proteinaceous product expressed by the sequence(s), as well as proteolytic fragments of such products. As such, the detection of the presence of, amount of, stability of, or degradation (including rate) of, such DNA, RNA and proteinaceous molecules may be used in the practice of the invention.

The practice of the present invention is unaffected by the presence of minor mismatches between the disclosed sequences and those expressed by cells of a subject’s sample. A non-limiting example of the existence of such mismatches are seen in cases of sequence polymorphisms between individuals of a species, such as individual human patients within *Homo sapiens*. Knowledge that expression of the disclosed sequences (and sequences that vary due to minor mismatches) is correlated with the presence of non-normal or abnormal breast cells and breast cancer is sufficient for the practice of the invention with an appropriate cell containing sample via an assay for expression.

In one embodiment, the invention provides for the identification of the expression levels of the disclosed sequences by analysis of their expression in a sample containing ER+ or ER- breast

cells. In one preferred embodiment, the sample contains single cells or homogenous cell populations which have been dissected away from, or otherwise isolated or purified from, contaminating cells beyond that possible by a simple biopsy. Alternatively, undissected cells within a "section" of tissue may be used. Multiple means for such analysis are available, including
5 detection of expression within an assay for global, or near global, gene expression in a sample (e.g. as part of a gene expression profiling analysis such as on a microarray) or by specific detection, such as quantitative PCR (Q-PCR), or real time quantitative PCR.

Preferably, the sample is isolated via non-invasive or minimally invasive means. The expression of the disclosed sequence(s) in the sample may be determined and compared to the
10 expression of said sequence(s) in reference data of non-normal or cancerous breast cells.

Alternatively, the expression level may be compared to expression levels in normal or non-cancerous cells, preferably from the same sample or subject. In embodiments of the invention utilizing Q-PCR, the expression level may be compared to expression levels of reference genes in the same sample or a ratio of expression levels may be used.

15 When individual breast cells are isolated in the practice of the invention, one benefit is that contaminating, non-breast cells (such as infiltrating lymphocytes or other immune system cells) are not present to possibly affect detection of expression of the disclosed sequence(s). Such contamination is present where a biopsy is used to generate gene expression profiles. However, analysis of differential gene expression and correlation to ER+ breast cancer outcomes with both
20 isolated and non-isolated samples, as described herein, increases the confidence level of the disclosed sequences as capable of having significant predictive power with either type of sample.

While the present invention is described mainly in the context of human breast cancer, it may be practiced in the context of breast cancer of any animal known to be potentially afflicted by breast cancer. Preferred animals for the application of the present invention are mammals,
25 particularly those important to agricultural applications (such as, but not limited to, cattle, sheep, horses, and other "farm animals"), animal models of breast cancer, and animals for human companionship (such as, but not limited to, dogs and cats).

The above aspects and embodiments of the invention may be applied equally with respect to use of more than one "antiestrogen" agent against breast cancer. In the case of a combination of

agents, any combination of more than one SERM, SERD, or AI may be used in place of TAM or another “antiestrogen” agent against breast cancer. Aromatase is an enzyme that provides a major source of estrogen in body tissues including the breast, liver, muscle and fat. Without being bound by theory, and solely provided to assist in a better understanding of the invention, AIs are

5 understood to function in a manner comparable to TAM and other “antiestrogen” agents against breast cancer, which are thought to act as antagonists of estrogen receptor in breast tissues and thus as against breast cancer. AIs may be either nonsteroidal or steroidal agents. Examples of the former, which inhibit aromatase via the heme prosthetic group) include, but are not limited to, anastrozole (arimidex), letrozole (femara), and vorozole (rivisor), which have been used or
10 contemplated as treatments for metastatic breast cancer. Examples of steroidal AIs, which inactivate aromatase, include, but are not limited to, exemestane (aromasin), androstenedione, and formestane (lentaron).

Other forms of therapy to reduce estrogen levels include surgical or chemical ovarian ablation. The former is physical removal of the ovaries while the latter is the use of agents to block
15 ovarian production of estrogen. One non-limiting example of the latter are agonists of gonadotropin releasing hormone (GnRH), such as goserelin (zoladex). Of course the instant invention may also be practiced with these therapies in place of treatment with one or more “antiestrogen” agent against breast cancer.

The invention disclosed herein is based in part on the performance of a genome-wide
20 microarray analysis of hormone receptor-positive invasive breast tumors from 60 patients treated with adjuvant tamoxifen alone, leading to the identification of a two-gene expression ratio that is highly predictive of clinical outcome. This expression ratio, which is readily adapted to PCR-based analysis of standard paraffin-embedded clinical specimens, was validated in an independent set of 20 patients as described below.

Brief Description of the Drawings

Figure 1 shows receiver operating characteristic (ROC) analyses of IL17BR, HOXB13, and CACNA1D expression levels as predictors of breast cancer outcomes in whole tissue sections (top 3 graphs) and laser microdissected cells (bottom 3 graphs). AUC refers to area under the curve.

5 Figure 2 contains six parts relating to the validation of a ratio of HOXB13 expression to IL17BR expression as an indicator of responsiveness, or lack thereof, to TAM. Parts a and b show the results of gene expression analysis of HOXB13 and IL17BR sequences by Q-PCR in both Responder and Non-responder samples. Plots of the Responder and Non-responder training and validation data sets are shown in Parts c and d, where “0” indicates Responder datapoints in both
10 and “1” indicates Non-responder datapoints in both. Parts e and f show plots of the Responder and Non-responder training and validation data sets as a function of survival, where the upper line in each Part represents the Responders and the lower line represents the Non-responders.

Modes of Practicing the Invention

Definitions of terms as used herein:

15 A gene expression “pattern” or “profile” or “signature” refers to the relative expression of genes correlated with responsiveness to treatment of ER+ breast cancer with TAM or another “antiestrogen” agent against breast cancer. Responsiveness or lack thereof may be expressed as survival outcomes which are correlated with an expression “pattern” or “profile” or “signature” that is able to distinguish between, and predict, said outcomes.

20 A “selective estrogen receptor modulator” or SERM is an “antiestrogen” agent that in some tissues act like estrogens (agonist) but block estrogen action in other tissues (antagonist). A “selective estrogen receptor downregulators” (or “SERD”s) or “pure” antiestrogens includes agents which block estrogen activity in all tissues. See Howell et al. (Best Practice & Res. Clin. Endocrinol. Metab. 18(1):47-66, 2004). Preferred SERMs of the invention are those that are
25 antagonists of estrogen in breast tissues and cells, including those of breast cancer. Non-limiting examples of such include TAM, raloxifene, GW5638, and ICI 182,780. The possible mechanisms of action by various SERMs have been reviewed (see for example Jordan et al., 2003, Breast Cancer Res. 5:281-283; Hall et al., 2001, J. Biol. Chem. 276(40):36869-36872; Dutertre et al. 2000, J.

Pharmacol. Exp. Therap. 295(2):431-437; and Wijayaratne et al., 1999, Endocrinology 140(12):5828-5840). Other non-limiting examples of SERMs in the context of the invention include triphenylethylenes, such as tamoxifen, GW5638, TAT-59, clomiphene, toremifene, droloxifene, and idoxifene; benzothiophenes, such as arzoxiphenes (LY353381 or LY353381-HCl);
5 benzopyrans, such as EM-800; naphthalenes, such as CP-336,156; and ERA-923.

Non-limiting examples of SERD or “pure” antiestrogens include agents such as ICI 182,780 (fulvestrant or faslodex) or the oral analogue SR16243 and ZK 191703 as well as aromatase inhibitors and chemical ovarian ablation agents as described herein.

Other agents encompassed by SERM as used herein include progesterone receptor inhibitors
10 and related drugs, such as progestomimetics like medroxyprogesterone acetate, megace, and RU-486; and peptide based inhibitors of ER action, such as LH-RH analogs (leuprolide, zoladex, [D-Trp6]LH-RH), somatostatin analogs, and LXXLL motif mimics of ER as well as tibolone and resveratrol. As noted above, preferred SERMs of the invention are those that are antagonist of estrogen in breast tissues and cells, including those of breast cancer. Non-limiting examples of
15 preferred SERMs include the actual or contemplated metabolites (in vivo) of any SERM, such as, but not limited to, 4-hydroxytamoxifen (metabolite of tamoxifen), EM652 (or SCH 57068 where EM-800 is a prodrug of EM-652), and GW7604 (metabolite of GW5638). See Willson et al. (1997, Endocrinology 138(9):3901-3911) and Dauvois et al. (1992, Proc. Nat’l. Acad. Sci., USA 89:4037-4041) for discussions of some specific SERMs.

20 Other preferred SERMs are those that produce the same relevant gene expression profile as tamoxifen or 4-hydroxytamoxifen. One example of means to identify such SERMs is provided by Levenson et al. (2002, Cancer Res. 62:4419-4426).

A “gene” is a polynucleotide that encodes a discrete product, whether RNA or proteinaceous in nature. It is appreciated that more than one polynucleotide may be capable of encoding a discrete
25 product. The term includes alleles and polymorphisms of a gene that encodes the same product, or a functionally associated (including gain, loss, or modulation of function) analog thereof, based upon chromosomal location and ability to recombine during normal mitosis.

A “sequence” or “gene sequence” as used herein is a nucleic acid molecule or polynucleotide composed of a discrete order of nucleotide bases. The term includes the ordering of

bases that encodes a discrete product (i.e. “coding region”), whether RNA or proteinaceous in nature, as well as the ordered bases that precede or follow a “coding region”. Non-limiting examples of the latter include 5’ and 3’ untranslated regions of a gene. It is appreciated that more than one polynucleotide may be capable of encoding a discrete product. It is also appreciated that alleles and polymorphisms of the disclosed sequences may exist and may be used in the practice of the invention to identify the expression level(s) of the disclosed sequences or the allele or polymorphism. Identification of an allele or polymorphism depends in part upon chromosomal location and ability to recombine during mitosis.

The terms “correlate” or “correlation” or equivalents thereof refer to an association between expression of one or more genes and a physiological response of a breast cancer cell and/or a breast cancer patient in comparison to the lack of the response. A gene may be expressed at higher or lower levels and still be correlated with responsiveness, non-responsiveness or breast cancer survival or outcome. The invention provides for the correlation between increases in expression of IL17BR and CACNA1D sequences and responsiveness of ER+ breast cells to TAM or another “antiestrogen” agent against breast cancer. Thus increases are indicative of responsiveness. Conversely, the lack of increases, including unchanged expression levels, are indicators of non-responsiveness. Similarly, the invention provides for the correlation between decreases in expression of HOXB13 sequences and responsiveness of ER+ breast cells to TAM or another SERM. Thus decreases are indicative of responsiveness while the lack of decreases, including unchanged expression levels, are indicators of non-responsiveness. Increases and decreases may be readily expressed in the form of a ratio between expression in a non-normal cell and a normal cell such that a ratio of one (1) indicates no difference while ratios of two (2) and one-half indicate twice as much, and half as much, expression in the non-normal cell versus the normal cell, respectively. Expression levels can be readily determined by quantitative methods as described below.

For example, increases in IL17BR, CACNA1D, or HOXB13 expression can be indicated by ratios of or about 1.1, of or about 1.2, of or about 1.3, of or about 1.4, of or about 1.5, of or about 1.6, of or about 1.7, of or about 1.8, of or about 1.9, of or about 2, of or about 2.5, of or about 3, of or about 3.5, of or about 4, of or about 4.5, of or about 5, of or about 5.5, of or about 6, of or about

6.5, of or about 7, of or about 7.5, of or about 8, of or about 8.5, of or about 9, of or about 9.5, of or about 10, of or about 15, of or about 20, of or about 30, of or about 40, of or about 50, of or about 60, of or about 70, of or about 80, of or about 90, of or about 100, of or about 150, of or about 200, of or about 300, of or about 400, of or about 500, of or about 600, of or about 700, of or about 800, of or about 900, of or about 1000. A ratio of 2 is a 100% (or a two-fold) increase in expression. Decreases in IL17BR, CACNA1D, or HOXB13 expression can be indicated by ratios of or about 0.9, of or about 0.8, of or about 0.7, of or about 0.6, of or about 0.5, of or about 0.4, of or about 0.3, of or about 0.2, of or about 0.1, of or about 0.05, of or about 0.01, of or about 0.005, of or about 0.001, of or about 0.0005, of or about 0.0001, of or about 0.00005, of or about 0.00001, of or about 0.000005, or of or about 0.000001.

For a given phenotype, a ratio of the expression of a gene sequence expressed at increased levels in correlation with the phenotype to the expression of a gene sequence expressed at decreased levels in correlation with the phenotype may also be used as an indicator of the phenotype. As a non-limiting example, the phenotype of non-responsiveness to tamoxifen treatment of breast cancer is correlated with increased expression of HOXB13 as well as decreased expression of IL17BR and CACNA1D. Therefore, a ratio of the expression levels of HOXB13 to IL17BR (or CACNA1D) may be used as an indicator of non-responsiveness.

A “polynucleotide” is a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA and RNA. It also includes known types of modifications including labels known in the art, methylation, “caps”, substitution of one or more of the naturally occurring nucleotides with an analog, and internucleotide modifications such as uncharged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), as well as unmodified forms of the polynucleotide.

The term “amplify” is used in the broad sense to mean creating an amplification product can be made enzymatically with DNA or RNA polymerases. “Amplification,” as used herein, generally refers to the process of producing multiple copies of a desired sequence, particularly those of a sample. “Multiple copies” mean at least 2 copies. A “copy” does not necessarily mean perfect sequence complementarity or identity to the template sequence. Methods for amplifying mRNA are

generally known in the art, and include reverse transcription PCR (RT-PCR) and those described in U.S. Patent Application 10/062,857 (filed on October 25, 2001), as well as U.S. Provisional Patent Applications 60/298,847 (filed June 15, 2001) and 60/257,801 (filed December 22, 2000), all of which are hereby incorporated by reference in their entireties as if fully set forth. Another method which may be used is quantitative PCR (or Q-PCR). Alternatively, RNA may be directly labeled as the corresponding cDNA by methods known in the art.

By "corresponding", it is meant that a nucleic acid molecule shares a substantial amount of sequence identity with another nucleic acid molecule. Substantial amount means at least 95%, usually at least 98% and more usually at least 99%, and sequence identity is determined using the BLAST algorithm, as described in Altschul et al. (1990), J. Mol. Biol. 215:403-410 (using the published default setting, i.e. parameters $w=4$, $t=17$).

A "microarray" is a linear or two-dimensional or three dimensional (and solid phase) array of preferably discrete regions, each having a defined area, formed on the surface of a solid support such as, but not limited to, glass, plastic, or synthetic membrane. The density of the discrete regions on a microarray is determined by the total numbers of immobilized polynucleotides to be detected on the surface of a single solid phase support, preferably at least about $50/\text{cm}^2$, more preferably at least about $100/\text{cm}^2$, even more preferably at least about $500/\text{cm}^2$, but preferably below about $1,000/\text{cm}^2$. Preferably, the arrays contain less than about 500, about 1000, about 1500, about 2000, about 2500, or about 3000 immobilized polynucleotides in total. As used herein, a DNA microarray is an array of oligonucleotides or polynucleotides placed on a chip or other surfaces used to hybridize to amplified or cloned polynucleotides from a sample. Since the position of each particular group of primers in the array is known, the identities of a sample polynucleotides can be determined based on their binding to a particular position in the microarray. As an alternative to the use of a microarray, an array of any size may be used in the practice of the invention, including an arrangement of one or more position of a two-dimensional or three dimensional arrangement in a solid phase to detect expression of a single gene sequence.

Because the invention relies upon the identification of genes that are over- or under-expressed, one embodiment of the invention involves determining expression by hybridization of mRNA, or an amplified or cloned version thereof, of a sample cell to a polynucleotide that is unique

to a particular gene sequence. Preferred polynucleotides of this type contain at least about 16, at least about 18, at least about 20, at least about 22, at least about 24, at least about 26, at least about 28, at least about 30, or at least about 32 consecutive basepairs of a gene sequence that is not found in other gene sequences. The term "about" as used in the previous sentence refers to an increase or decrease of 1 from the stated numerical value. Even more preferred are polynucleotides of at least or about 50, at least or about 100, at least or about 150, at least or about 200, at least or about 250, at least or about 300, at least or about 350, at least or about 400, , at least or about 450, or at least or about 500 consecutive bases of a sequence that is not found in other gene sequences. The term "about" as used in the preceding sentence refers to an increase or decrease of 10% from the stated numerical value. Longer polynucleotides may of course contain minor mismatches (e.g. via the presence of mutations) which do not affect hybridization to the nucleic acids of a sample. Such polynucleotides may also be referred to as polynucleotide probes that are capable of hybridizing to sequences of the genes, or unique portions thereof, described herein. Such polynucleotides may be labeled to assist in their detection. Preferably, the sequences are those of mRNA encoded by the genes, the corresponding cDNA to such mRNAs, and/or amplified versions of such sequences. In preferred embodiments of the invention, the polynucleotide probes are immobilized on an array, other solid support devices, or in individual spots that localize the probes.

In another embodiment of the invention, all or part of a disclosed sequence may be amplified and detected by methods such as the polymerase chain reaction (PCR) and variations thereof, such as, but not limited to, quantitative PCR (Q-PCR), reverse transcription PCR (RT-PCR), and real-time PCR (including as a means of measuring the initial amounts of mRNA copies for each sequence in a sample), optionally real-time RT-PCR or real-time Q-PCR. Such methods would utilize one or two primers that are complementary to portions of a disclosed sequence, where the primers are used to prime nucleic acid synthesis. The newly synthesized nucleic acids are optionally labeled and may be detected directly or by hybridization to a polynucleotide of the invention. The newly synthesized nucleic acids may be contacted with polynucleotides (containing sequences) of the invention under conditions which allow for their hybridization. Additional methods to detect the expression of expressed nucleic acids include RNase protection assays, including liquid phase hybridizations, and *in situ* hybridization of cells.

Alternatively, and in yet another embodiment of the invention, gene expression may be determined by analysis of expressed protein in a cell sample of interest by use of one or more antibodies specific for one or more epitopes of individual gene products (proteins), or proteolytic fragments thereof, in said cell sample or in a bodily fluid of a subject. The cell sample may be one of breast cancer epithelial cells enriched from the blood of a subject, such as by use of labeled antibodies against cell surface markers followed by fluorescence activated cell sorting (FACS). Such antibodies are preferably labeled to permit their easy detection after binding to the gene product. Detection methodologies suitable for use in the practice of the invention include, but are not limited to, immunohistochemistry of cell containing samples or tissue, enzyme linked immunosorbent assays (ELISAs) including antibody sandwich assays of cell containing tissues or blood samples, mass spectroscopy, and immuno-PCR.

The term “label” refers to a composition capable of producing a detectable signal indicative of the presence of the labeled molecule. Suitable labels include radioisotopes, nucleotide chromophores, enzymes, substrates, fluorescent molecules, chemiluminescent moieties, magnetic particles, bioluminescent moieties, and the like. As such, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means.

The term “support” refers to conventional supports such as beads, particles, dipsticks, fibers, filters, membranes and silane or silicate supports such as glass slides.

As used herein, a “breast tissue sample” or “breast cell sample” refers to a sample of breast tissue or fluid isolated from an individual suspected of being afflicted with, or at risk of developing, breast cancer. Such samples are primary isolates (in contrast to cultured cells) and may be collected by any non-invasive or minimally invasive means, including, but not limited to, ductal lavage, fine needle aspiration, needle biopsy, the devices and methods described in U.S. Patent 6,328,709, or any other suitable means recognized in the art. Alternatively, the “sample” may be collected by an invasive method, including, but not limited to, surgical biopsy.

“Expression” and “gene expression” include transcription and/or translation of nucleic acid material.

As used herein, the term “comprising” and its cognates are used in their inclusive sense; that is, equivalent to the term “including” and its corresponding cognates.

Conditions that “allow” an event to occur or conditions that are “suitable” for an event to occur, such as hybridization, strand extension, and the like, or “suitable” conditions are conditions that do not prevent such events from occurring. Thus, these conditions permit, enhance, facilitate, and/or are conducive to the event. Such conditions, known in the art and described herein, depend upon, for example, the nature of the nucleotide sequence, temperature, and buffer conditions. These conditions also depend on what event is desired, such as hybridization, cleavage, strand extension or transcription.

Sequence “mutation,” as used herein, refers to any sequence alteration in the sequence of a gene disclosed herein interest in comparison to a reference sequence. A sequence mutation includes single nucleotide changes, or alterations of more than one nucleotide in a sequence, due to mechanisms such as substitution, deletion or insertion. Single nucleotide polymorphism (SNP) is also a sequence mutation as used herein. Because the present invention is based on the relative level of gene expression, mutations in non-coding regions of genes as disclosed herein may also be assayed in the practice of the invention.

“Detection” includes any means of detecting, including direct and indirect detection of gene expression and changes therein. For example, “detectably less” products may be observed directly or indirectly, and the term indicates any reduction (including the absence of detectable signal). Similarly, “detectably more” product means any increase, whether observed directly or indirectly.

Increases and decreases in expression of the disclosed sequences are defined in the following terms based upon percent or fold changes over expression in normal cells. Increases may be of 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, or 200% relative to expression levels in normal cells. Alternatively, fold increases may be of 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, or 10 fold over expression levels in normal cells. Decreases may be of 10, 20, 30, 40, 50, 55, 60, 65, 70, 75, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99 or 100% relative to expression levels in normal cells.

Unless defined otherwise all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs.

Embodiments of the Invention

In a first aspect, the disclosed invention relates to the identification and use of gene expression patterns (or profiles or “signatures”) which discriminate between (or are correlated with) breast cancer survival in a subject treated with tamoxifen (TAM) or another “antiestrogen” agent against breast cancer. Such patterns may be determined by the methods of the invention by use of a number of reference cell or tissue samples, such as those reviewed by a pathologist of ordinary skill in the pathology of breast cancer, which reflect breast cancer cells as opposed to normal or other non-cancerous cells. The outcomes experienced by the subjects from whom the samples may be correlated with expression data to identify patterns that correlate with the outcomes following treatment with TAM or another “antiestrogen” agent against breast cancer. Because the overall gene expression profile differs from person to person, cancer to cancer, and cancer cell to cancer cell, correlations between certain cells and genes expressed or underexpressed may be made as disclosed herein to identify genes that are capable of discriminating between breast cancer outcomes.

The present invention may be practiced with any number of the genes believed, or likely to be, differentially expressed with respect to breast cancer outcomes, particularly in cases of ER+ breast cancer. The identification may be made by using expression profiles of various homogenous breast cancer cell populations, which were isolated by microdissection, such as, but not limited to, laser capture microdissection (LCM) of 100-1000 cells. The expression level of each gene of the expression profile may be correlated with a particular outcome. Alternatively, the expression levels of multiple genes may be clustered to identify correlations with particular outcomes.

Genes with significant correlations to breast cancer survival when the subject is treated with tamoxifen may be used to generate models of gene expressions that would maximally discriminate between outcomes where a subject responds to treatment with tamoxifen or another “antiestrogen” agent against breast cancer and outcomes where the treatment is not successful. Alternatively, genes with significant correlations may be used in combination with genes with lower correlations without significant loss of ability to discriminate between outcomes. Such models may be generated by any appropriate means recognized in the art, including, but not limited to, cluster analysis, supported vector machines, neural networks or other algorithm known in the art. The

models are capable of predicting the classification of a unknown sample based upon the expression of the genes used for discrimination in the models. "Leave one out" cross-validation may be used to test the performance of various models and to help identify weights (genes) that are uninformative or detrimental to the predictive ability of the models. Cross-validation may also be used to identify genes that enhance the predictive ability of the models.

The gene(s) identified as correlated with particular breast cancer outcomes relating to tamoxifen treatment by the above models provide the ability to focus gene expression analysis to only those genes that contribute to the ability to identify a subject as likely to have a particular outcome relative to another. The expression of other genes in a breast cancer cell would be relatively unable to provide information concerning, and thus assist in the discrimination of, a breast cancer outcome.

As will be appreciated by those skilled in the art, the models are highly useful with even a small set of reference gene expression data and can become increasingly accurate with the inclusion of more reference data although the incremental increase in accuracy will likely diminish with each additional datum. The preparation of additional reference gene expression data using genes identified and disclosed herein for discriminating between different outcomes in breast cancer following treatment with tamoxifen or another "antiestrogen" agent against breast cancer is routine and may be readily performed by the skilled artisan to permit the generation of models as described above to predict the status of an unknown sample based upon the expression levels of those genes.

To determine the (increased or decreased) expression levels of genes in the practice of the present invention, any method known in the art may be utilized. In one preferred embodiment of the invention, expression based on detection of RNA which hybridizes to the genes identified and disclosed herein is used. This is readily performed by any RNA detection or amplification+detection method known or recognized as equivalent in the art such as, but not limited to, reverse transcription-PCR, the methods disclosed in U.S. Patent Application 10/062,857 (filed on October 25, 2001) as well as U.S. Provisional Patent Applications 60/298,847 (filed June 15, 2001) and 60/257,801 (filed December 22, 2000), and methods to detect the presence, or absence, of RNA stabilizing or destabilizing sequences.

Alternatively, expression based on detection of DNA status may be used. Detection of the DNA of an identified gene as methylated or deleted may be used for genes that have decreased expression in correlation with a particular breast cancer outcome. This may be readily performed by PCR based methods known in the art, including, but not limited to, Q-PCR. Conversely,
5 detection of the DNA of an identified gene as amplified may be used for genes that have increased expression in correlation with a particular breast cancer outcome. This may be readily performed by PCR based, fluorescent *in situ* hybridization (FISH) and chromosome *in situ* hybridization (CISH) methods known in the art.

Expression based on detection of a presence, increase, or decrease in protein levels or
10 activity may also be used. Detection may be performed by any immunohistochemistry (IHC) based, blood based (especially for secreted proteins), antibody (including autoantibodies against the protein) based, exfoliate cell (from the cancer) based, mass spectroscopy based, and image (including used of labeled ligand) based method known in the art and recognized as appropriate for the detection of the protein. Antibody and image based methods are additionally useful for the
15 localization of tumors after determination of cancer by use of cells obtained by a non-invasive procedure (such as ductal lavage or fine needle aspiration), where the source of the cancerous cells is not known. A labeled antibody or ligand may be used to localize the carcinoma(s) within a patient or to assist in the enrichment of exfoliated cancer cells from a bodily fluid.

A preferred embodiment using a nucleic acid based assay to determine expression is by
20 immobilization of one or more sequences of the genes identified herein on a solid support, including, but not limited to, a solid substrate as an array or to beads or bead based technology as known in the art. Alternatively, solution based expression assays known in the art may also be used. The immobilized gene(s) may be in the form of polynucleotides that are unique or otherwise specific to the gene(s) such that the polynucleotide would be capable of hybridizing to a DNA or
25 RNA corresponding to the gene(s). These polynucleotides may be the full length of the gene(s) or be short sequences of the genes (up to one nucleotide shorter than the full length sequence known in the art by deletion from the 5' or 3' end of the sequence) that are optionally minimally interrupted (such as by mismatches or inserted non-complementary basepairs) such that hybridization with a DNA or RNA corresponding to the gene(s) is not affected. Preferably, the polynucleotides used are

from the 3' end of the gene, such as within about 350, about 300, about 250, about 200, about 150, about 100, or about 50 nucleotides from the polyadenylation signal or polyadenylation site of a gene or expressed sequence. Polynucleotides containing mutations relative to the sequences of the disclosed genes may also be used so long as the presence of the mutations still allows hybridization to produce a detectable signal.

The immobilized gene(s) may be used to determine the state of nucleic acid samples prepared from sample breast cell(s) for which the outcome of the sample's subject (e.g. patient from whom the sample is obtained) is not known or for confirmation of an outcome that is already assigned to the sample's subject. Without limiting the invention, such a cell may be from a patient with ER+ or ER- breast cancer. The immobilized polynucleotide(s) need only be sufficient to specifically hybridize to the corresponding nucleic acid molecules derived from the sample under suitable conditions. While even a single correlated gene sequence may be able to provide adequate accuracy in discriminating between two breast cancer outcomes, two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, or eleven or more of the genes identified herein may be used as a subset capable of discriminating may be used in combination to increase the accuracy of the method. The invention specifically contemplates the selection of more than one, two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, or eleven or more of the genes disclosed in the tables and figures herein for use as a subset in the identification of breast cancer survival outcome.

Of course 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, or all the genes provided in Tables 2 and/or 3 below may be used. "Accession" as used in the context of the Tables herein as well as the present invention refers to the GenBank accession number of a sequence of each gene, the sequences of which are hereby incorporated by reference in their entireties as they are available from GenBank as accessed on the filing date of the present application. P value refers to values assigned as described in the Examples below. The indications of "E-xx" where "xx" is a two digit number refers to alternative notation for exponential figures where "E-xx" is " 10^{-xx} ". Thus in combination with the numbers to the left of "E-xx", the

value being represented is the numbers to the left times 10^{-xx} . "Description" as used in the Tables provides a brief identifier of what the sequence/gene encodes.

Genes with a correlation identified by a p value below or about 0.02, below or about 0.01, below or about 0.005, or below or about 0.001 are preferred for use in the practice of the invention.

5 The present invention includes the use of gene(s) the expression of which identify different breast cancer outcomes after treatment with TAM or another "antiestrogen" agent against breast cancer to permit simultaneous identification of breast cancer survival outcome of a patient based upon assaying a breast cancer sample from said patient.

10 In a second aspect, the present invention relates to the identification and use of three sets of sequences for the determination of responsiveness of ER+ breast cancer to treatment with TAM or another "antiestrogen" agent against breast cancer. The differential expression of these sequences in breast cancer relative to normal breast cells is used to predict responsiveness to TAM or another "antiestrogen" agent against breast cancer in a subject.

15 To identify gene expression patterns in ER positive, early stage invasive breast cancers that might predict response to hormonal therapy, microarray gene expression analysis was performed on tumors from 60 women uniformly treated with adjuvant tamoxifen alone. These patients were identified from a total of 103 ER+ early stage cases presenting to Massachusetts General Hospital between 1987 and 1997, from whom tumor specimens were snap frozen and for whom minimal 5 year follow-up was available (see Table 1 for details). Within this cohort, 28 (46%) women
20 developed distant metastasis with a median time to recurrence of 4 years ("tamoxifen non-responders") and 32 (54%) women remained disease-free with median follow-up of 10 years ("tamoxifen responders"). Responders were matched with non-responder cases with respect to TNM staging (see Singletary, S.E. et al. "Revision of the American Joint Committee on Cancer staging system for breast cancer." J Clin Oncol 20, 3628-36 (2002)) and tumor grade (see Dalton,
25 L.W. et al. "Histologic grading of breast cancer: linkage of patient outcome with level of pathologist agreement." Mod Pathol 13, 730-5. (2000)).

Previous studies linking gene expression profiles to clinical outcome in breast cancer have demonstrated that the potential for distant metastasis and overall survival probability may be predictable through biological characteristics of the primary tumor at the time of diagnosis (see

Huang, E. et al. "Gene expression predictors of breast cancer outcomes." Lancet 361, 1590-6 (2003); Sorlie, T. et al. "Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications." Proc Natl Acad Sci U S A 98:10869-74 (2001); Sorlie, T. et al. "Repeated observation of breast tumor subtypes in independent gene expression data sets." Proc Natl Acad Sci U S A 100, 8418-23 (2003); Sotiriou, C. et al. "Breast cancer classification and prognosis based on gene expression profiles from a population-based study." Proc Natl Acad Sci U S A 100, 10393-8 (2003); van 't Veer, L.J. et al. "Gene expression profiling predicts clinical outcome of breast cancer." Nature 415, 530-6 (2002); and van de Vijver, M.J. et al. "A gene-expression signature as a predictor of survival in breast cancer." N Engl J Med 347, 1999-2009 (2002)). In particular, a 70-gene expression signature has proven to be a strong prognostic factor, out-performing all known clinicopathological parameters. However, in those studies patients either received no adjuvant therapy (van 't Veer, L.J. et al. Nature 2002) or were treated non-uniformly with hormonal and chemotherapeutic regimens (Huang, E. et al.; Sorlie, T. et al.; Sorlie, T. et al.; Sotiriou, C. et al.; and van de Vijver, M.J. et al. N Engl J Med 2002). Patients with ER+ early-stage breast cancer treated with tamoxifen alone, such as the cohort studied here, represent only a subset of the population tested with the 70-gene signature. Of note, 61 of the genes in the 70-gene signature were present on the microarray used as described below, but no significant association with clinical outcome was observed in the defined subset of patients.

In comparison with existing biomarkers, including ESR1, PGR, ERBB2 and EGFR, three sets of gene sequences disclosed herein are significantly more predictive of responsiveness to TAM treatment. Multivariate analysis indicated that these three genes were significant predictors of clinical outcome independent of tumor size, nodal status and tumor grade. ER and progesterone receptor (PR) expression have been the major clinicopathological predictors for response to TAM. However, up to 40% of ER+ tumors fail to respond or develop resistance to TAM. The invention thus provides for the use of the identified biomarkers to allow better patient management by identifying patients who are more likely to benefit from TAM or other endocrine therapy and those who are likely to develop resistance and tumor recurrence.

As noted herein, the sequences(s) identified by the present invention are expressed in correlation with ER+ breast cancer cells. For example, IL17BR, identified by I.M.A.G.E.

Consortium Clusters NM_018725 and NM_172234 ("The I.M.A.G.E. Consortium: An Integrated Molecular Analysis of Genomes and their Expression," Lennon et al., 1996, Genomics 33:151-152; see also image.llnl.gov) has been found to be useful in predicting responsiveness to TAM treatment.

In preferred embodiments of the invention, any sequence, or unique portion thereof, of the
5 IL17BR sequences of the cluster, as well as the UniGene *Homo sapiens* cluster Hs.5470, may be used. Similarly, any sequence encoding all or a part of the protein encoded by any IL17BR sequence disclosed herein may be used. Consensus sequences of I.M.A.G.E. Consortium clusters are as follows, with the assigned coding region (ending with a termination codon) underlined and preceded by the 5' untranslated and/or non-coding region and followed by the 3' untranslated
10 and/or non-coding region:

SEQ ID NO:1 (consensus sequence for IL17BR, transcript variant 1, identified as NM_018725 or NM_018725.2)

15 agcgcagcgt gcggttgcc tggatcccgc gcagtggccc ggcgatgtcg ctcggtgtgc
taagcctggc cgcgtgtgc aggagcgccg taccgagaga gccgaccgtt caatgtggct
ctgaaactgg gccatctcca gagtggatgc tacaacatga tctaattccc ggagacttga
gggacctccg agtagaacct gttacaacta gtgttgcaac aggggactat tcaattttga
tgaatgtaag ctgggtactc cgggcagatg ccagcatccg cttgttgaag gccaccaaga
20 tttgtgtgac gggcaaaagc aacttccagt cctacagctg tgtgaggtgc aattacacag
aggccttcca gactcagacc agaccctctg gtggtaaagt gacattttcc tacatcggct
tccctgtaga gctgaacaca gtctatttca ttggggccca taatattcct aatgcaaata
tgaatgaaga tggcccttcc atgtctgtga atttcacctc accaggctgc ctagaccaca
taatgaaata taaaaaaaag tgtgtcaagg ccggaagcct gtgggatccg aacatcactg
25 cttgtaagaa gaatgaggag acagtagaag tgaacttcac aaccactccc ctgggaagaa
gatacatggc tcttatccaa cacagcacta tcatcgggtt ttctcaggtg tttgagccac
accagaagaa acaaacgcga gcttcagtgg tgattccagt gactggggat agtgaagggtg
ctacgggtgca gctgactcca tattttcccta cttgtggcag cgactgcata cgacataaag
gaacagttgt gctctgcccc caaacaggcg tccctttccc tctggataac aacaaaagca
30 agccgggagg ctggctgcct ctctctctgc tgtctctgct ggtggccaca tgggtgctgg
tggcagggat ctatctaagt tggaggcacg aaaggatcaa gaagacttcc ttttctacca
ccacactact gccccccatt aaggttcttg tggtttacc ctttgaaata tgtttccatc
acacaatttg ttacttcact gaatttcttc aaaaccattg cagaagtgag gtcactcttg
aaaagtggca gaaaaagaaa atagcagaga tgggtccagt gcagtggctt gccactcaaa
35 agaaggcagc agacaaagtc gtcttccttc tttccaatga cgtcaacagt gtgtgcatg
gtacctgtgg caagagcgag ggcagtccca gtgagaactc tcaagacctc tcccccttg
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ttttaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaa

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SEQ ID NO:2 (consensus sequence for IL17BR, transcript variant 2, identified as NM_172234 or NM_172234.1)

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15  gccacccat gagaagcaag agaccttaaa ggcttcctat cccaccaatt acagggaaaa
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    aactacattt acaacttcaa agctgtttta tacatagaaa tcaattacag ttttaattga
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20  catagaccat gcattgcagt gtaccagaa ctgttttagct aatattctat gtttaattaa
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    ttctattaca tgcaaaaaat cattgttttt aagataacaa aagtagggaa taaacaagct
    gaaccactt ttaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaa

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25 I.M.A.G.E. Consortium Clone ID numbers and the corresponding GenBank accession
 numbers of sequences identified as belonging to the I.M.A.G.E. Consortium and UniGene clusters,
 are listed below. Also included are sequences that are not identified as having a Clone ID number
 but still identified as being those of IL17BR. The sequences include those of the "sense" and
 complementary strands sequences corresponding to IL17BR. The sequence of each GenBank
 30 accession number is presented in the attached Appendix.

Clone ID numbers	GenBank accession numbers
2985728	AW675096, AW673932, BC000980
5286745	BI602183
5278067	BI458542
5182255	BI823321
924000	AA514396
3566736	BF110326

3195409	BE466508
3576775	BF740045
2772915	AW299271
1368826	AA836217
1744837	AI203628
2285564	AI627783
2217709	AI744263
2103651	AI401622
2419487	AI826949
3125592	BE047352
2284721	AI911549
3643302	BF194822
1646910	AI034244
1647001	AI033911
3323709	BF064177
1419779	AA847767
2205190	AI538624
2295838	AI913613
2461335	AI942234
2130362	AI580483
2385555	AI831909
2283817	AI672344
2525596	AW025192
454687	AA677205
1285273	AA721647
3134106	BF115018
342259	W61238, W61239
1651991	AI032064
2687714	AW236941
3302808	BG057174
2544461	AW058532
122014	T98360, T98361
2139250	AI470845

2133899	AI497731
121300	T96629, T96740
162274	H25975, H25941
3446667	BE539514, BX282554
156864	R74038, R74129
4611491	BG433769
4697316	BG530489
429376	AA007528, AA007529
5112415	BI260259
701357	AA287951, AA287911
121909	T97852, T97745
268037	N40294
1307489	AA809841
1357543	AA832389
48442	H14692
1302619	AA732635
1562857	AA928257
1731938	AI184427
1896025	AI298577
2336350	AI692717
1520997	AA910922
240506	H90761
2258560	AI620122
1569921	AI793318, AA962325, AI733290
6064627	BQ226353
299018	W04890
5500181	BM455231
2484011	BI492426
4746376	BG674622
233783	BX111256
1569921	BX117618
450450	AA682806
1943085	AI202376

2250390	AI658949
4526156	BG403405
3249181	BE673417
2484395	AW021469
30515867	CF455736
2878155	AW339874
4556884	BG399724
3254505	BF475787
3650593	BF437145
233783	H64601
None (mRNA sequences)	AF212365, AF208110, AF208111, AF250309, AK095091
None	BM983744, CB305764, BM715988, BM670929, BI792416, BI715216, N56060, CB241389, AV660618, BX088671, CB154426, CA434589, CA412162, CA314073, BF921554, BF920093, AV685699, AV650175, BX483104, CD675121, BE081436, AW970151, AW837146, AW368264, D25960, AV709899, BX431018, AL535617, AL525465, BX453536, BX453537, AV728945, AV728939, AV727345

In one preferred embodiment, any sequence, or unique portion thereof, of the following IL17BR sequence, identified by AF208111 or AF208111.1, may be used in the practice of the invention.

5

SEQ ID NO:3 (sequence for IL17BR):

10 CGGCGATGTCGCTCGTGCTGATAAGCCTGGCCGCGCTGTGCAGGAGCGCCGTACCCCGAG
AGCCGACCGTTCAATGTGGCTCTGAACTGGGCCATCTCCAGAGTGGATGCTACAACATG
ATCTAATCCCGGAGACTTGAGGGACCTCCGAGTAGAACCTGTTACAAC TAGTGTGCAA
CAGGGGACTATTCAATTTTGATGAATGTAAGCTGGGTACTCCGGGCAGATGCCAGCATCC
GCTTGTTGAAGGCCACCAAGATTTGTGTGACGGGCAAAAGCAACTTCCAGTCCTACAGCT
GTGTGAGGTGCAATTACACAGAGGCCCTTCCAGACTCAGACCAGACCCTCTGGTGGTAAAT
15 GGACATTTTCCTATATCGGCTTCCCTGTAGAGCTGAACACAGTCTATTTTCATTGGGGCCC
ATAATATTCCTAATGCAAATATGAATGAAGATGGCCCTTCCATGTCTGTGAATTTACCT
CACCAGGCTGCCTAGACCACATAATGAAATATAAAAAAAGTGTGTCAAGGCCGGAAGCC

TGTGGGATCCGAACATCACTGCTTGTAAGAAGAATGAGGAGACAGTAGAAGTGAACCTCA
CAACCACTCCCCTGGGAAACAGATACATGGCTCTTATCCAACACAGCACTATCATCGGGT
TTTCTCAGGTGTTTGAGCCACACCAGAAGAAACAAACGCGAGCTTCAGTGGTGATTCCAG
TGACTGGGGATAGTGAAGGTGCTACGGTGCAGGTAAAGTTCAGTGAGCTGCTCTGGGGAG
5 GGAAGGGACATAGAAGACTGTTCCATCATTCATTGCTTTTAAGGATGAGTTCTCTCTTGT
CAAATGCACTTCTGCCAGCAGACACCAGTTAAGTGGCGTTCATGGGGGTCTTTTCGCTGC
AGCCTCCACCGTGCTGAGGTCAGGAGGCCGACGTGGCAGTTGTGGTCCCTTTTGTCTGTA
TTAATGGCTGCTGACCTTCCAAAGCACTTTTTATTTTCATTTTCTGTACAGACACTCAG
GGATAGCAGTACCATTTTACTTCCGCAAGCCTTTAACTGCAAGATGAAGCTGCAAAGGGT
10 TTGAAATGGGAAGGTTTGAGTTCAGGCAGCGTATGAACTCTGGAGAGGGGCTGCCAGTC
CTCTCTGGGCCGACGCGGACCCAGCTGGAACACAGGAAGTTGGAGCAGTAGGTGCTCCTT
CACCTCTCAGTATGTCTCTTTCAACTCTAGTTTTTGAAGTGGGGACACAGGAAGTCCAGT
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AGTGNTCAAACACACCAGAGGGGGCAGGCACCAGCCAGGGTATGATGGGTACTACCCTTT
15 TCTGGAGAACCATAGACTTCCCTTACTACAGGGACTTGCATGTCCTAAAGCACTGGCTGA
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AAGATATGACCTAGCCCTTTTAGGTAAGCGAACTGGTATGTTAGTAACGTGTACAAAGTT
TAGGTTTCAGACCCCGGGAGTCTTGGGCATGTGGGTCTCGGGTCACTGGTTTTGACTTTAG
GGCTTTGTTACAGATGTGTGACCAAGGGGAAAATGTGCATGACAACACTAGAGGTAGGGG
20 CGAAGCCAGAAAGAAGGGAAGTTTTGGCTGAAGTAGGAGTCTTGGTGAGATTTTGCTGTG
ATGCATGGTGTGAACTTTCTGAGCCTCTTGTTTTTCTCAGCTGACTCCATATTTTCCTA
CTTGTGGCAGCGACTGCATCCGACATAAAGGAACAGTTGTGCTCTGCCCACAAACAGGCG
TCCCTTTTCCCTCTGGATAACAACAAAAGCAAGCCGGGAGGCTGGCTGCCTCTCCTCCTGC
TGTCTCTGCTGGTGGCCACATGGGTGCTGGTGGCAGGGATCTATCTAATGTGGAGGCACG
25 AAAGGATCAAGAAGACTTCCTTTTCTACCACCACACTACTGCCCCCATTAAGGTTCTTG
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TTTCCAATGACGTCAACAGTGTGTGCGATGGTACCTGTGGCAAGAGCGAGGGCAGTCCCA
30 GTGAGAACTCTCAAGACCTCTTCCCCCTTGCCCTTTAACCTTTTCTGCAGTGATCTAAGAA
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GTGCAGAACTTCTCCATGTCAAGCAGCAGGTGTCAGCAGGAAAAAGATCACAAGCCTGCC
ACGATGGCTGCTGCTCCTTGTAGCCCAACCATGAGAAGCAAGAGACCTTAAAGGCTTCCT
35 ATCCCACCAATTACAGGGAAAAAACGTGTGATGATCCTGAAGCTTACTATGCAGCCTACA
AACAGCCTTAGTAATTAACCATTTTATACCAATAAAATTTTCAAATATTACTAACTAAT
GTAGCATTAACCTAACGATTGGAACTACATTTACAACCTTCAAAGCTGTTTTATACATAGA
AATCAATTACAGCTTTAATTGAAAACGTGAACCATTTTGATAATGCAACAATAAAGCATC
TTCCAAAAA

40

In another set of preferred embodiments of the invention, any sequence, or unique portion thereof, of the CACNA1D sequences of the I.M.A.G.E. Consortium cluster NM_000720, as well as

the UniGene Homo sapiens cluster Hs.399966, may be used. Similarly, any sequence encoding all or a part of the protein encoded by any CACNA1D sequence disclosed herein may be used. The consensus sequence of the I.M.A.G.E. Consortium cluster is as follows, with the assigned coding region (ending with a termination codon) underlined and preceded by the 5' untranslated and/or non-coding region and followed by the 3' untranslated and/or non-coding region:

SEQ ID NO:4 (consensus sequence for CACNA1D, identified as NM_000720 or NM_000720.1)

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10  agaataaggg cagggaccgc ggctcctatc tcttggtgat ccccttcccc attccgcccc
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    gatgatgatg atgatgatga aaaaaatgca gcatcaacgg cagcagcaag cggaccacgc
    gaacgaggca aactatgcaa gaggcaccag acttcctctt tctggtgaag gaccaacttc
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acggaaagaa caaggactgg tgggaaagta ccctgcgaag aacaccacaa ttgccctaca
ggcgggatta aggacactgc atgacattgg gccagaaatc cggcgtgcta tatcgtgtga
tttgcaagat gacgagcctg aggaacaaaa acgagaagaa gaagatgatg tgttcaaaaag
10 aaatgggtgcc ctgcttgga accatgtcaa tcatgttaat agtgatagga gagattccct
tcagcagacc aataccaccc accgtccctc gcatgtccaa aggccttcaa ttccacctgc
aagtgatact gagaaaccgc tgtttcctcc agcaggaaat tcggtgtgtc ataaccatca
taaccataat tccataggaa agcaagttcc cacctcaaca aatgccaatc tcaataatgc
caatatgtcc aaagctgccc atggaaagcg gcccagcatt gggaaccttg agcatgtgtc
15 tgaaaatggg catcattctt cccacaagca tgaccgggag cctcagagaa ggtccagtgt
gaaaagaacc cgctattatg aaacttacat taggtccgac tcaggagatg aacagctccc
aactatttgc cgggaagacc cagagataca tggctatttc agggaccccc actgcttggg
ggagcaggag tatttcagta gtgaggaatg ctacgaggat gacagctcgc ccacctggag
caggcaaaac tatggctact acagcagata ccaggcaga aacatcgact ctgagaggcc
20 ccgaggctac catcatcccc aaggattctt ggaggacgat gactcgcccc tttgctatga
ttcacggaga tctccaagga gacgcctact acctcccacc ccagcatccc accggagatc
ctccttcaac tttgagtgcc tgcgccggca gagcagccag gaagagggtcc cgctgtctcc
catcttcccc catcgcacgg ccctgcctct gcatctaata cagcaacaga tcatggcagt
tgccggccta gattcaagta aagcccagaa gtactaccg agtcactcga cccggtcgtg
25 ggccacccct ccagcaaccc ctccctaccg ggactggaca ccgtgctaca cccccctgat
ccaagtggag cagtcagagg ccctggacca ggtgaacggc agcctgccgt ccctgcaccg
cagctcctgg tacacagacg agcccagat ctccctaccg actttcacac cagccagcct
gactgtcccc agcagcttcc ggaacaaaaa cagcgacaag cagaggagtg cggacagctt
gggtggaggca gtccctgatat ccgaaggctt gggacgctat gcaagggacc caaaatttgt
30 gtcagcaaca aaacacgaaa tcgctgatgc ctgtgacctc accatcgacg agatggagag
tgcagccagc accctgctta atgggaacgt gcgtccccga gccaacgggg atgtgggccc
cctctcacac cggcaggact atgagctaca ggactttgggt cctggctaca gcgacgaaga
gccagaccct gggaggggatg aggaggacct ggccgatgaa atgatatgca tcaccacctt
gtagccccca gcgaggggca gactggctct ggccctcaggt ggggcgcagg agagccaggg
35 gaaaagtgcc tcatagttag gaaagttag gactagttag ggagtaatat tcaattaatt
agacttttgt ataagagatg tcatgcctca agaaagccat aaacctggta ggaacaggtc
ccaagcgggt gagcctggca gagtaccatg cgctcgcccc cagctgcagg aaacagcagg
ccccgcctc tcacagagga tgggtgagga ggccagacct gccctgcccc attgtccaga
tgggcaactgc tgtggagtct gcttctccca tgtaccaggg caccaggccc acccaactga
40 aggcattggcg gcgggggtgca ggggaaagt aaaggtgatg acgatcatca cacctcgtgt
cgttacctca gccatcggtc tagcatatca gtactgggc ccaacatatc catttttaaa
ccctttcccc caaatacact gcgtcctgggt tcctgttttag ctgttctgaa ata

I.M.A.G.E. Consortium Clone ID numbers and the corresponding GenBank accession numbers of sequences identified as belonging to the I.M.A.G.E. Consortium and UniGene clusters, are listed below. Also included are sequences that are not identified as having a Clone ID number but still identified as being those of CACNA1D. The sequences include those of the "sense" and complementary strands sequences corresponding to CACNA1D. The sequence of each GenBank accession number is presented in the attached Appendix.

Clone ID numbers	GenBank accession numbers
5676430	BM128550
5197948	BI755471
6027638	BQ549084, BQ549571
2338956	AI693324
36581	R25307, R46658
49630	H29256, H29339
4798765	BG716371
2187310	AI537488
838231	AA458692
2111614	AI393327
2183482	AI520947
1851007	AI248998
1675503	AI075844
2434923	AI869807
2434924	AI869800
1845827	AI243110
2511756	AI955764
628568	AA192669, AA192157
2019331	AI361691
2337381	AI914244
2503579	AW008769
2503626	AW008794
1160989	AA877582
1653475	AI051972

1627755	AI017959
287750	N79331, N62240
1867677	AI240933
1618303	AI015031
1881344	AI290994
1408031	AA861160
1557035	AA915941
956303	AA493341
2148234	AI467998
1499899	AA885585
1647592	AI033648
2341185	AI697633
981603	AA523647
6281678	BQ710377
6278348	BQ706920
5876024	BQ016847
6608849	CA943595
5440464	BM008196
5209489	BI769856
5183025	BI758971
880540	AA468565
757337	AA437099
6608849	CA867864
461797	AA682690
434787	AA701888
6151588	BU182632
6295618	BQ898429
6300779	BQ711800
434811	AA703120
1568025	AA978315
3220210	BE550599
3214121	BE502741
3009312	AW872382

2733394	AW444663
2872156	AW341279
30514550	CF456750
2718456	AW139850
2543682	AW029633
2492730	AI963788
2545866	AI951788
2272081	AI680744
2152336	AI601252
2146429	AI459166
1274498	AA885750
2272081	BX092736
287750	BX114568
3233645	BE672659
289209	N78509, N73668
277086	N46744, N39597
3272340	BF439267
3273859	BF436153
3568401	BF110611
None (mRNA sequences)	M76558, AF088004, M83566
None	CB410657, BQ372430, BQ366601, BQ324528, BQ318830, AL708030, BM509161, N85902, BQ774355, CA774243, CA436347, CA389011, BU679327, BU608029, BU073743, BE175413, AW969248, AI908115, BF754485, BI015409, BG202552, BF883669, BF817590, BF807128, BF806160, BF805244, BF805235, BF805080, T27949, BE836638, BE770685, BE769065,

In one preferred embodiment, any sequence, or unique portion thereof, of the following CACNA1D sequence, identified by AF088004 or AF088004.1, may be used in the practice of the invention.

5

SEQ ID NO:5 (sequence for CACNA1D):

TTTTTTTTTTTTTTTTTTTTCTTACAAAGAAAAATTTAATATTCGATGAGAGGTTGAAC
CAGGCTTAAAGCAGACATACTAGGAAATGGTGCAGCCTGTAAGAATGCCAGTTTGTAAGT
ACTGACTTTGGAAAAGATCATCGCCTCTATCAGACACTTAGGGTCCTGGTCTGGCAATTT
5 TGGCCTGATGTGATGCCACAAGACCCAACAGAGAGAGACACAGAGTCCAGGATAATGTTG
ACAGTGGTGTAGCCCTTTAGGAGAAATGGCGCTCCCTGCGGCTGGTATTAGGTTACCATT
GGCACCGAAGGAACCAGGAGGATAAGAATATCCATAATTTTCAGAGCTGCCCTGGCACAGT
ACCTGCCCCGTCGGAGGCTCTCACTGGCAAATGACAGCTCTGTGCAAGGAGCACTCCCAA
GTATAAAAATTATTACACAGTTTTATTCTGAAGAACATTTTGCATTTTAATAAAAAAGGA
10 TTTATGTCAGGAAAGAGTCATTTACAAACCTTGAAGTGTTTTTGCCTGGATCAGAGTAAG
AATGTCTTAAGAAGAGGTTTGTAAGGTCTTCATAACAAAGTGGTGTTTGTATTATTACAAA
AAAAAAAAAAAAAAAAAATTAACAGGTTGTCTGTATACTATTAAAAATTTTGGACCAAAAA
AAAAAAAAAAAAAAAAA

15 In another set of preferred embodiments of the invention, any sequence, or unique portion thereof, of the HOXB13 sequences of the I.M.A.G.E. Consortium cluster NM_006361, as well as the UniGene Homo sapiens cluster Hs.66731, may be used. Similarly, any sequence encoding all or a part of the protein encoded by any HOXB13 sequence disclosed herein may be used. The consensus sequence of the I.M.A.G.E. Consortium cluster is as follows, with the assigned coding
20 region (ending with a termination codon) underlined and preceded by the 5' untranslated and/or non-coding region and followed by the 3' untranslated and/or non-coding region:

SEQ ID NO:6 (consensus sequence for HOXB13, identified as NM_006361 or NM_006361.2)

25 cgaatgcagg cgacttgcca gctgggagcg atttaaaacg ctttggattc ccccggcctg
ggtggggaga gcgagctggg tgccccctag attccccgcc cccgcacctc atgagccgac
cctcggctcc atggagcccg gcaattatgc caccttggat ggagccaagg atatcgaagg
cttgctggga gcgggagggg ggcggaatct ggtcgccac tccctctga ccagccaccc
agcggcgccct acgctgatgc ctgctgtcaa ctatgcccc ttggatctgc caggctcggc
30 ggagccgcca aagcaatgcc acccatgccc tggggtgccc caggggacgt cccagctcc
cgtgccttat ggttactttg gaggcgggta ctactcctgc cgagtgtccc ggagctcgt
gaaaccctgt gccaggcag ccacctggc gcggtacccc gcggagactc ccacggccgg
ggaagagtac ccagtcgcc cactgagtt tgccttctat ccgggatatc cgggaacct
ccacgctatg gccagttacc tggacgtgtc tgtggtgcag actctgggtg ctctggaga
35 accgcgacat gactccctgt tgctgtgga cagttaccag tcttgggctc tcgctggtgg
ctggaacagc cagatgtgtt gccagggaga acagaacca ccaggccct tttggaaggc
agcatttgca gactccagcg ggcagcacc tctgacgcc tgcgcctttc gtcgcgccg
caagaaacgc attccgtaca gcaaggggca gttgcgggag ctggagcggg agtatgcggc
taacaagttc atcaccaagg acaagaggcg caagatctcg gcagccacca gcctctcgg

5 gcgccagatt accatctggt ttcagaaccg ccgggtcaaa gagaagaagg ttctcgccaa
ggtgaagaac agcgctaccc cttagagat ctccttgctt ggggtggagg agcgaaagt
ggggtgtcct ggggagacca gaaacctgcc aagcccaggc tggggccaag gactctgctg
agaggccct agagacaaca cccttcccag gccactggct gctggactgt tcctcaggag
cggcctgggt acccagtatg tgcagggaga cggaaccca tgtgacaggc ccactccacc
agggttcca aagaacctgg ccagtcata atcattcatc ctcacagtgg caataatcac
gataaccagt

10 I.M.A.G.E. Consortium Clone ID numbers and the corresponding GenBank accession
numbers of sequences identified as belonging to the I.M.A.G.E. Consortium and UniGene clusters,
are listed below. Also included are sequences that are not identified as having a Clone ID number
but still identified as being those of HOXB13. The sequences include those of the "sense" and
complementary strands sequences corresponding to HOXB13. The sequence of each GenBank
accession number is presented in the attached Appendix.

15

Clone ID numbers	GenBank accession numbers
4250486	BF676461, BC007092
5518335	BM462617
4874541	BG752489
4806039	BG778198
3272315	CB050884, CB050885
4356740	BF965191
6668163	BU930208
1218366	AA807966
2437746	AI884491
1187697	AA652388
3647557	BF446158
1207949	AA657924
1047774	AA644637
3649397	BF222357
971664	AA527613
996191	AA533227
813481	AA456069, AA455572, BX117624
6256333	BQ673782

2408470	AI814453
2114743	AI417272
998548	AA535663
2116027	AI400493
3040843	AW779219
1101311	AA594847
1752062	AI150430
898712	AA494387
1218874	AA662643
2460189	AI935940
986283	AA532530
1435135	AA857572
1871750	AI261980
3915135	BE888751
2069668	AI378797
667188	AA234220, AA236353
1101561	AA588193
1170268	AI821103, AI821851, AA635855
2095067	AI420753
4432770	BG180547
783296	AA468306, AA468232
3271646	CB050115, CB050116
1219276	AA661819
30570598	CF146837
30570517	CF146763
30568921	CF144902
3099071	CF141511
3096992	CF139563
3096870	CF139372
3096623	CF139319
3096798	CF139275
30572408	CF122893
2490082	AI972423

2251055	AI918975
2419308	AI826991
2249105	AI686312
2243362	AI655923
30570697	CF146922
3255712	BF476369
3478356	BF057410
3287977	BE645544
3287746	BE645408
3621499	BE388501
30571128	CF147366
30570954	CF147143
None (mRNA sequences)	BT007410, BC007092, U57052, U81599
None	CB120119, CB125764, AU098628, CB126130, BI023924, BM767063, BM794275, BQ363211, BM932052, AA357646, AW609525, CB126919, AW609336, AW609244, BF855145, AU126914, CB126449, AW582404, BX641644

In one preferred embodiment, any sequence, or unique portion thereof, of the following HOXB13 sequence, identified by BC007092 or BC007092.1, may be used in the practice of the invention.

5

SEQ ID NO:7 (sequence for HOXB13):

10 GGATTCCCCCGGCCTGGGTGGGGAGAGCGAGCTGGGTGCCCCCTAGATTCCCCGCCCCCG
 CACCTCATGAGCCGACCCTCGGCTCCATGGAGCCCGGAATTATGCCACCTTGGATGGAG
 CCAAGGATATCGAAGGCTTGCTGGGAGCGGGAGGGGGCGGAATCTGGTCGCCCCACTCCC
 CTCTGACCAGCCACCCAGCGGCGCCTACGCTGATGCCTGCTGTCAACTATGCCCCCTTGG
 ATCTGCCAGGCTCGGCGGAGCCGCCAAAGCAATGCCACCCATGCCCTGGGGTGCCCCAGG
 GGACGTCCCCAGCTCCCGTGCCCTTATGGTTACTTTGGAGGCGGGTACTACTCCTGCCGAG
 15 TGTCCCGGAGCTCGCTGAAACCCTGTGCCCAGGCAGCCACCCTGGCCGCGTACCCCGCGG
 AGACTCCACGGCCGGGAAGAGTACCCAGCCGCCCCACTGAGTTTGCCTTCTATCCGG
 GATATCCGGGAACCTACCAGCCTATGGCCAGTTACCTGGACGTGTCTGTGGTGACAGTCTT
 TGGGTGCTCCTGGAGAACC GCGACATGACTCCCTGTTGCCTGTGGACAGTTACCA GTCTT
 GGGCTCTCGCTGGTGGCTGGAACAGCCAGATGTGTTGCCAGGGAGAACAGAACCCACCAG

GTCCCTTTTGGGAAGGCAGCATTTGCAGACTCCAGCGGGCAGCACCCCTCCTGACGCCTGCG
CCTTTCGTCGCGGCCGCAAGAAACGCATTCCGTACAGCAAGGGGCAGTTGCGGGAGCTGG
AGCGGGAGTATGCGGCTAACAAGTTCATCACCAAGGACAAGAGGCGCAAGATCTCGGCAG
CCACCAGCCTCTCGGAGCGCCAGATTACCATCTGGTTTCAGAACCGCCGGGTCAAAGAGA
5 AGAAGGTTCTCGCCAAGGTGAAGAACAGCGCTACCCCTTAAGAGATCTCCTTGCCTGGGT
GGGAGGAGCGAAAGTGGGGGTGTCCTGGGGAGACCAGGAACCTGCCAAGCCCAGGCTGGG
GCCAAGGACTCTGCTGAGAGGCCCTAGAGACAACACCCTTCCCAGGCCACTGGCTGCTG
GACTGTTCCCTCAGGAGCGGCCTGGGTACCCAGTATGTGCAGGGAGACGGAACCCCATGTG
ACAGCCCACTCCACCAGGGTTCCCAAAGAACCTGGCCCAGTCATAATCATTCATCCTGAC
10 AGTGGCAATAATCACGATAACCAGTACTAGCTGCCATGATCGTTAGCCTCATATTTTCTA
TCTAGAGCTCTGTAGAGCACTTTAGAAACCGCTTTCATGAATTGAGCTAATTATGAATAA
ATTTGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Sequences identified by SEQ ID NO. are provided using conventional representations of a
15 DNA strand starting from the 5' phosphate linked end to the 3' hydroxyl linked end. The
assignment of coding regions is generally by comparison to available consensus sequence(s) and
therefore may contain inconsistencies relative to other sequences assigned to the same cluster.
These have no effect on the practice of the invention because the invention can be practiced by use
of shorter segments (or combinations thereof) of sequences unique to each of the three sets
20 described above and not affected by inconsistencies. As non-limiting examples, a segment of
IL17BR, CACNA1D, or HOXB13 nucleic acid sequence composed of a 3' untranslated region
sequence and/or a sequence from the 3' end of the coding region may be used as a probe for the
detection of IL17BR, CACNA1D, or HOXB13 expression, respectively, without being affected by
the presence of any inconsistency in the coding regions due to differences between sequences.
25 Similarly, the use of an antibody which specifically recognizes IL17BR, CACNA1D, or HOXB13
protein to detect its expression would not be affected by the presence of any inconsistency in the
representation of the coding regions provided above.

As will be appreciated by those skilled in the art, some of the above sequences include 3'
poly A (or poly T on the complementary strand) stretches that do not contribute to the uniqueness of
30 the disclosed sequences. The invention may thus be practiced with sequences lacking the 3' poly A
(or poly T) stretches. The uniqueness of the disclosed sequences refers to the portions or entireties
of the sequences which are found only in IL17BR, CACNA1D, or HOXB13 nucleic acids,
including unique sequences found at the 3' untranslated portion of the genes. Preferred unique
sequences for the practice of the invention are those which contribute to the consensus sequences

for each of the three sets such that the unique sequences will be useful in detecting expression in a variety of individuals rather than being specific for a polymorphism present in some individuals. Alternatively, sequences unique to an individual or a subpopulation may be used. The preferred unique sequences are preferably of the lengths of polynucleotides of the invention as discussed
5 herein.

To determine the (increased or decreased) expression levels of the above described sequences in the practice of the present invention, any method known in the art may be utilized. In one preferred embodiment of the invention, expression based on detection of RNA which hybridizes to polynucleotides containing the above described sequences is used. This is readily
10 performed by any RNA detection or amplification+detection method known or recognized as equivalent in the art such as, but not limited to, reverse transcription-PCR (optionally real-time PCR), the methods disclosed in U.S. Patent Application 10/062,857 entitled "Nucleic Acid Amplification" filed on October 25, 2001 as well as U.S. Provisional Patent Applications 60/298,847 (filed June 15, 2001) and 60/257,801 (filed December 22, 2000), the methods disclosed
15 in U.S. Patent 6,291,170, and quantitative PCR. Methods to identify increased RNA stability (resulting in an observation of increased expression) or decreased RNA stability (resulting in an observation of decreased expression) may also be used. These methods include the detection of sequences that increase or decrease the stability of mRNAs containing the IL17BR, CACNA1D, or HOXB13 sequences disclosed herein. These methods also include the detection of increased
20 mRNA degradation.

In particularly preferred embodiments of the invention, polynucleotides having sequences present in the 3' untranslated and/or non-coding regions of the above disclosed sequences are used to detect expression or non-expression of IL17BR, CACNA1D, or HOXB13 sequences in breast cells in the practice of the invention. Such polynucleotides may optionally contain sequences found
25 in the 3' portions of the coding regions of the above disclosed sequences. Polynucleotides containing a combination of sequences from the coding and 3' non-coding regions preferably have the sequences arranged contiguously, with no intervening heterologous sequence(s).

Alternatively, the invention may be practiced with polynucleotides having sequences present in the 5' untranslated and/or non-coding regions of IL17BR, CACNA1D, or HOXB13 sequences in

breast cells to detect their levels of expression. Such polynucleotides may optionally contain sequences found in the 5' portions of the coding regions. Polynucleotides containing a combination of sequences from the coding and 5' non-coding regions preferably have the sequences arranged contiguously, with no intervening heterologous sequence(s). The invention may also be practiced with sequences present in the coding regions of IL17BR, CACNA1D, or HOXB13.

Preferred polynucleotides contain sequences from 3' or 5' untranslated and/or non-coding regions of at least about 16, at least about 18, at least about 20, at least about 22, at least about 24, at least about 26, at least about 28, at least about 30, at least about 32, at least about 34, at least about 36, at least about 38, at least about 40, at least about 42, at least about 44, or at least about 46 consecutive nucleotides. The term "about" as used in the previous sentence refers to an increase or decrease of 1 from the stated numerical value. Even more preferred are polynucleotides containing sequences of at least or about 50, at least or about 100, at least or about 150, at least or about 200, at least or about 250, at least or about 300, at least or about 350, or at least or about 400 consecutive nucleotides. The term "about" as used in the preceding sentence refers to an increase or decrease of 10% from the stated numerical value.

Sequences from the 3' or 5' end of the above described coding regions as found in polynucleotides of the invention are of the same lengths as those described above, except that they would naturally be limited by the length of the coding region. The 3' end of a coding region may include sequences up to the 3' half of the coding region. Conversely, the 5' end of a coding region may include sequences up the 5' half of the coding region. Of course the above described sequences, or the coding regions and polynucleotides containing portions thereof, may be used in their entirety.

Polynucleotides combining the sequences from a 3' untranslated and/or non-coding region and the associated 3' end of the coding region are preferably at least or about 100, at least or about 150, at least or about 200, at least or about 250, at least or about 300, at least or about 350, or at least or about 400 consecutive nucleotides. Preferably, the polynucleotides used are from the 3' end of the gene, such as within about 350, about 300, about 250, about 200, about 150, about 100, or about 50 nucleotides from the polyadenylation signal or polyadenylation site of a gene or expressed sequence. Polynucleotides containing mutations relative to the sequences of the disclosed genes

may also be used so long as the presence of the mutations still allows hybridization to produce a detectable signal.

In another embodiment of the invention, polynucleotides containing deletions of nucleotides from the 5' and/or 3' end of the above disclosed sequences may be used. The deletions are preferably of 1-5, 5-10, 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-45, 45-50, 50-60, 60-70, 70-80, 80-90, 90-100, 100-125, 125-150, 150-175, or 175-200 nucleotides from the 5' and/or 3' end, although the extent of the deletions would naturally be limited by the length of the disclosed sequences and the need to be able to use the polynucleotides for the detection of expression levels.

Other polynucleotides of the invention from the 3' end of the above disclosed sequences include those of primers and optional probes for quantitative PCR. Preferably, the primers and probes are those which amplify a region less than about 350, less than about 300, less than about 250, less than about 200, less than about 150, less than about 100, or less than about 50 nucleotides from the from the polyadenylation signal or polyadenylation site of a gene or expressed sequence.

In yet another embodiment of the invention, polynucleotides containing portions of the above disclosed sequences including the 3' end may be used in the practice of the invention. Such polynucleotides would contain at least or about 50, at least or about 100, at least about or 150, at least or about 200, at least or about 250, at least or about 300, at least or about 350, or at least or about 400 consecutive nucleotides from the 3' end of the disclosed sequences.

The invention thus also includes polynucleotides used to detect IL17BR, CACNA1D, or HOXB13 expression in breast cells. The polynucleotides may comprise a shorter polynucleotide consisting of sequences found in the above provided SEQ ID NOS in combination with heterologous sequences not naturally found in combination with IL17BR, CACNA1D, or HOXB13 sequences.

As non-limiting examples, a polynucleotide comprising one of the following sequences may be used in the practice of the invention.

SEQ ID NO:8:

CAATTACAGGGAAAAACGTGTGATGATCCTGAAGCTTACTATGCAGCCTACAAACAGCC

SEQ ID NO:9:

GCTCTCACTGGCAAATGACAGCTCTGTGCAAGGAGCACTCCCAAGTATAAAAATTATTAC

SEQ ID NO:10:

GATCGTTAGCCTCATATTTTCTATCTAGAGCTCTGTAGAGCACTTTAGAAACCGCTTTCA

5

Stated differently, the invention may be practiced with a polynucleotide consisting of the sequence of SEQ ID NOS:8, 9 or 10 in combination with one or more heterologous sequences that are not normally found with SEQ ID NOS:8, 9 or 10. Alternatively, the invention may also be practiced with a polynucleotide consisting of the sequence of SEQ ID NOS:8, 9 or 10 in combination with one or more naturally occurring sequences that are normally found with SEQ ID NOS:8, 9 or 10.

10

Polynucleotides with sequences comprising SEQ ID NOS:8 or 9, either naturally occurring or synthetic, may be used to detect nucleic acids which are over expressed in breast cancer cells that are responsive, and those which are not over expressed in breast cancer cells that are non-responsive, to treatment with TAM or another “antiestrogen” agent against breast cancer. Polynucleotides with sequences comprising SEQ ID NO:10, either naturally occurring or synthetic, may be used to detect nucleic acids which are under expressed in breast cancer cells that are responsive, and those which are not under expressed in breast cancer cells that are non-responsive, to treatment with TAM or another “antiestrogen” agent against breast cancer.

15

20

Additional sequences that may be used in polynucleotides as described above for SEQ ID NOS:8 and 9 are the following, wherein SEQ ID NOS:33 is complementary to a portion of IL17BR sequences disclosed herein:

SEQ ID NO:11:

25

TGCCTAATTTCACTCTCAGAGTGAGGCAGGTAAGTGGGGCTCCACTGGGTCACTCTGAGA

SEQ ID NO:12:

TTGGAAGCAGAGTCCCTCTAAAGGTAAGTCTTGTGGTCACTCAATATTGTATTGGCATT

30

SEQ ID NO:13:

ACGTTAGACTTTTGCTGGCATTCAAGTCATGGCTAGTCTGTGTATTTAATAAATGTGTGT

SEQ ID NO:14:

CTGGTCAGCCACTCTGACTTTTCTACCACATTAAATTCTCCATTACATCTCACTATTGGT

SEQ ID NO:15:

5 TACAACCTTCTGAATGCTGCACATTCTTCCAAAATGATCCTTAGCACAATCTATTGTATGA

SEQ ID NO:16:

GGGATGGCCTTTAGGCCACAGTAGTGTCTGTGTTAAGTTCATAAATGTGTATTTAATGA

SEQ ID NO:17:

10 CTCAAAGTGCTAAAGCTATGGTTGACTGCTCTGGTGTTTTTATATTCATTCGTGCTTTAG

SEQ ID NO:32:

15 CTGAAGCTTACTATGCAGCCTACAA

SEQ ID NO:33:

TCCAATCGTTAGTTAATGCTACATTAGTT

SEQ ID NO:34:

20 CAGCCTTAGTAATTAAAAC

Additional sequences that may be used in polynucleotides as described above for SEQ ID NO:10 are the following, wherein SEQ ID NOs:36 is complementary to a portion of IL17BR sequences disclosed herein:

SEQ ID NO:18:

25 CTATGGGGATGGTCCACTGTCACTGTTTCTCTGCTGTTGCAAATACATGGATAACACATT

SEQ ID NO:19:

30 ACTGGAAGAGCAGATGGTCTGACTGTGCTATGGCCTCATCATCAAGACTTTCAATCCTAT

SEQ ID NO:20:

ACGCCAAGCTCTTCAGTGAAGACACGATGTTATTAAAAGCCTGTTTTAGGGACTGCAAAA

SEQ ID NO:21:

35 TTTTGTAAAATCTTTAACCTTCCCTTTGTTCTTCATGTACACGCTGAACTGCAATTCTT

SEQ ID NO:22:

AACCTGGGGCATTTAGGGCAGAGGACAAAAGGATGTCAGCAATTGCTTGGGCTGCTTGGC

SEQ ID NO:23:

5 CTGGAACCTCTGGACTCCCCATGCTCTAACTCCCACACTCTGCTATCAGAACTTAACT

SEQ ID NO:24:

AACCCAGAACCATCTAAGACATGGGATTCAGTGATCATGTGGTTCTCCTTTTAACTTAC

SEQ ID NO:25:

10 GGCCATGTGCCATGGTATTTGGGTCCTGGGAGGGTGGGTGAAATAAAGGCATACTGTCTT

SEQ ID NO:26:

15 GTGTAGGCAGTCATGGCACCAAAGCCACCAGACTGACAAATGTGTATCAGATGCTTTTGT

SEQ ID NO:27:

GAAAACCTCTTCAAAAGACAAAAGCTGGCACTGCATTCTCTCTGTAGCAGGACAGAA

SEQ ID NO:28:

20 CACATCTTTAGGGTCAGTGAACAATGGGGCACATTTGGCACTAGCTTGAGCCCAACTCTG

SEQ ID NO:29:

GCCTTAATTTCTCATCTGAAAAGCTGGAAGGCCTGACTTGACTTGTTGAGCTTAAGATCC

SEQ ID NO:30:

25 CTTCAGGGGAGGATCAAGCTTTGAACCAAAGCCAATCACTGGCTTGATTTGTGTTTTTTA

SEQ ID NO:31:

30 ACAAGTTTTCACTGAATGAGCATGGCAGTGCCACTCAAGAAAATGAATCTCCAAAGTATC

SEQ ID NO:35:

GCCATGATCGTTAGCCTCATATT

SEQ ID NO:36:

35 CAATTCATGAAAGCGGTTTCTAAAG

SEQ ID NO:37:

TCTATCTAGAGCTCTGTAGAGC

Additionally, polynucleotides containing other sequences, particularly unique sequences, present in naturally occurring nucleic acid molecules comprising SEQ ID NOS:8-37 may be used in the practice of the invention.

Other polynucleotides for use in the practice of the invention include those that have
5 sufficient homology to those described above to detect expression by use of hybridization techniques. Such polynucleotides preferably have about or 95%, about or 96%, about or 97%, about or 98%, or about or 99% identity with IL17BR, CACNA1D, or HOXB13 sequences as described herein. Identity is determined using the BLAST algorithm, as described above. The other polynucleotides for use in the practice of the invention may also be described on the basis of
10 the ability to hybridize to polynucleotides of the invention under stringent conditions of about 30% v/v to about 50% formamide and from about 0.01M to about 0.15M salt for hybridization and from about 0.01M to about 0.15M salt for wash conditions at about 55 to about 65°C or higher, or conditions equivalent thereto.

In a further embodiment of the invention, a population of single stranded nucleic acid
15 molecules comprising one or both strands of a human IL17BR or CACNA1D sequence is provided as a probe such that at least a portion of said population may be hybridized to one or both strands of a nucleic acid molecule quantitatively amplified from RNA of a breast cancer cell. The population may be only the antisense strand of a human IL17BR or CACNA1D sequence such that a sense strand of a molecule from, or amplified from, a breast cancer cell may be hybridized to a portion of
20 said population. The population preferably comprises a sufficiently excess amount of said one or both strands of a human IL17BR or CACNA1D sequence in comparison to the amount of expressed (or amplified) nucleic acid molecules containing a complementary IL17BR or CACNA1D sequence from a normal breast cell. This condition of excess permits the increased amount of nucleic acid expression in a breast cancer cell to be readily detectable as an increase.

25 Alternatively, the population of single stranded molecules is equal to or in excess of all of one or both strands of the nucleic acid molecules amplified from a breast cancer cell such that the population is sufficient to hybridize to all of one or both strands. Preferred cells are those of a breast cancer patient that is ER+ or for whom treatment with tamoxifen or one or more other “antiestrogen” agent against breast cancer is contemplated. The single stranded molecules may of

course be the denatured form of any IL17BR and/or CACNA1D sequence containing double stranded nucleic acid molecule or polynucleotide as described herein.

The population may also be described as being hybridized to IL17BR or CACNA1D sequence containing nucleic acid molecules at a level of at least twice as much as that by nucleic acid molecules of a normal breast cell. As in the embodiments described above, the nucleic acid molecules may be those quantitatively amplified from a breast cancer cell such that they reflect the amount of expression in said cell.

The population is preferably immobilized on a solid support, optionally in the form of a location on a microarray. A portion of the population is preferably hybridized to nucleic acid molecules quantitatively amplified from a non-normal or abnormal breast cell by RNA amplification. The amplified RNA may be that derived from a breast cancer cell, as long as the amplification used was quantitative with respect to IL17BR or CACNA1D containing sequences.

In another embodiment of the invention, expression based on detection of DNA status may be used. Detection of the HOXB13 DNA as methylated, deleted or otherwise inactivated, may be used as an indication of decreased expression as found in non-normal breast cells. This may be readily performed by PCR based methods known in the art. The status of the promoter regions of HOXB13 may also be assayed as an indication of decreased expression of HOXB13 sequences. A non-limiting example is the methylation status of sequences found in the promoter region.

Conversely, detection of the DNA of a sequence as amplified may be used for as an indication of increased expression as found in non-normal breast cells. This may be readily performed by PCR based, fluorescent *in situ* hybridization (FISH) and chromosome *in situ* hybridization (CISH) methods known in the art.

A preferred embodiment using a nucleic acid based assay to determine expression is by immobilization of one or more of the sequences identified herein on a solid support, including, but not limited to, a solid substrate as an array or to beads or bead based technology as known in the art. Alternatively, solution based expression assays known in the art may also be used. The immobilized sequence(s) may be in the form of polynucleotides as described herein such that the polynucleotide would be capable of hybridizing to a DNA or RNA corresponding to the sequence(s).

The immobilized polynucleotide(s) may be used to determine the state of nucleic acid samples prepared from sample breast cancer cell(s), optionally as part of a method to detect ER status in said cell(s). Without limiting the invention, such a cell may be from a patient suspected of being afflicted with, or at risk of developing, breast cancer. The immobilized polynucleotide(s) need only be sufficient to specifically hybridize to the corresponding nucleic acid molecules derived from the sample (and to the exclusion of detectable or significant hybridization to other nucleic acid molecules).

In yet another embodiment of the invention, a ratio of the expression levels of two of the disclosed genes may be used to predict response to treatment with TAM or another SERM.

Preferably, the ratio is that of two genes with opposing patterns of expression, such as an underexpressed gene to an overexpressed gene, in correlation to the same phenotype. Non-limiting examples include the ratio of HOXB13 over IL17BR or the ratio of HOXB13 over CACNA1D. This aspect of the invention is based in part on the observation that such a ratio has a stronger correlation with TAM treatment outcome than the expression level of either gene alone. For example, the ratio of HOXB13 over IL17BR has an observed classification accuracy of 77%.

As a non-limiting example, the Ct values from Q-PCR based detection of gene expression levels may be used to derive a ratio to predict the response to treatment with one or more “antiestrogen” agent against breast cancer.

Additional Embodiments of the Invention

In embodiments where only one or a few genes are to be analyzed, the nucleic acid derived from the sample breast cancer cell(s) may be preferentially amplified by use of appropriate primers such that only the genes to be analyzed are amplified to reduce contaminating background signals from other genes expressed in the breast cell. Alternatively, and where multiple genes are to be analyzed or where very few cells (or one cell) is used, the nucleic acid from the sample may be globally amplified before hybridization to the immobilized polynucleotides. Of course RNA, or the cDNA counterpart thereof may be directly labeled and used, without amplification, by methods known in the art.

Sequence expression based on detection of a presence, increase, or decrease in protein levels or activity may also be used. Detection may be performed by any immunohistochemistry (IHC)

based, bodily fluid based (where a IL17BR, CACNA1D, and/or HOXB13 polypeptide is found in a bodily fluid, such as but not limited to blood), antibody (including autoantibodies against the protein where present) based, ex foliate cell (from the cancer) based, mass spectroscopy based, and image (including used of labeled ligand where available) based method known in the art and
5 recognized as appropriate for the detection of the protein. Antibody and image based methods are additionally useful for the localization of tumors after determination of cancer by use of cells obtained by a non-invasive procedure (such as ductal lavage or fine needle aspiration), where the source of the cancerous cells is not known. A labeled antibody or ligand may be used to localize the carcinoma(s) within a patient.

10 Antibodies for use in such methods of detection include polyclonal antibodies, optionally isolated from naturally occurring sources where available, and monoclonal antibodies, including those prepared by use of IL17BR, CACNA1D, and/or HOXB13 polypeptides as antigens. Such antibodies, as well as fragments thereof (including but not limited to F_{ab} fragments) function to detect or diagnose non-normal or cancerous breast cells by virtue of their ability to specifically bind
15 IL17BR, CACNA1D, or HOXB13 polypeptides to the exclusion of other polypeptides to produce a detectable signal. Recombinant, synthetic, and hybrid antibodies with the same ability may also be used in the practice of the invention. Antibodies may be readily generated by immunization with a IL17BR, CACNA1D, or HOXB13 polypeptide, and polyclonal sera may also be used in the practice of the invention.

20 Antibody based detection methods are well known in the art and include sandwich and ELISA assays as well as Western blot and flow cytometry based assays as non-limiting examples. Samples for analysis in such methods include any that contain IL17BR, CACNA1D, or HOXB13 polypeptides. Non-limiting examples include those containing breast cells and cell contents as well as bodily fluids (including blood, serum, saliva, lymphatic fluid, as well as mucosal and other
25 cellular secretions as non-limiting examples) that contain the polypeptides.

 The above assay embodiments may be used in a number of different ways to identify or detect the response to treatment with TAM or another “antiestrogen” agent against breast cancer based on gene expression in a breast cancer cell sample from a patient. In some cases, this would reflect a secondary screen for the patient, who may have already undergone mammography or

physical exam as a primary screen. If positive from the primary screen, the subsequent needle biopsy, ductal lavage, fine needle aspiration, or other analogous minimally invasive method may provide the sample for use in the assay embodiments before, simultaneous with, or after assaying for ER status. The present invention is particularly useful in combination with non-invasive
5 protocols, such as ductal lavage or fine needle aspiration, to prepare a breast cell sample.

The present invention provides a more objective set of criteria, in the form of gene expression profiles of a discrete set of genes, to discriminate (or delineate) between breast cancer outcomes. In particularly preferred embodiments of the invention, the assays are used to discriminate between good and poor outcomes after treatment with tamoxifen or another
10 “antiestrogen” agent against breast cancer. Comparisons that discriminate between outcomes after about 10, about 20, about 30, about 40, about 50, about 60, about 70, about 80, about 90, about 100, or about 150 months may be performed.

While good and poor survival outcomes may be defined relatively in comparison to each other, a “good” outcome may be viewed as a better than 50% survival rate after about 60 months
15 post surgical intervention to remove breast cancer tumor(s). A “good” outcome may also be a better than about 60%, about 70%, about 80% or about 90% survival rate after about 60 months post surgical intervention. A “poor” outcome may be viewed as a 50% or less survival rate after about 60 months post surgical intervention to remove breast cancer tumor(s). A “poor” outcome may also be about a 70% or less survival rate after about 40 months, or about a 80% or less survival rate after
20 about 20 months, post surgical intervention.

In another embodiment of the invention based on the expression of a few genes, the isolation and analysis of a breast cancer cell sample may be performed as follows:

- (1) Ductal lavage or other non-invasive procedure is performed on a patient to obtain a sample.
- (2) Sample is prepared and coated onto a microscope slide. Note that ductal lavage results in
25 clusters of cells that are cytologically examined as stated above.
- (3) Pathologist or image analysis software scans the sample for the presence of atypical cells.
- (4) If atypical cells are observed, those cells are harvested (e.g. by microdissection such as LCM).
- (5) RNA is extracted from the harvested cells.

(6) RNA is assayed, directly or after conversion to cDNA or amplification therefrom, for the expression of IL17BR, CACNA1D, and/or HOXB13 sequences.

With use of the present invention, skilled physicians may prescribe or withhold treatment with TAM or another “antiestrogen” agent against breast cancer based on prognosis determined via practice of the instant invention.

The above discussion is also applicable where a palpable lesion is detected followed by fine needle aspiration or needle biopsy of cells from the breast. The cells are plated and reviewed by a pathologist or automated imaging system which selects cells for analysis as described above.

The present invention may also be used, however, with solid tissue biopsies, including those stored as an FFPE specimen. For example, a solid biopsy may be collected and prepared for visualization followed by determination of expression of one or more genes identified herein to determine the breast cancer outcome. As another non-limiting example, a solid biopsy may be collected and prepared for visualization followed by determination of HOXB13, IL17BR and/or CACNA1D expression. One preferred means is by use of *in situ* hybridization with polynucleotide or protein identifying probe(s) for assaying expression of said gene(s).

In an alternative method, the solid tissue biopsy may be used to extract molecules followed by analysis for expression of one or more gene(s). This provides the possibility of leaving out the need for visualization and collection of only cancer cells or cells suspected of being cancerous.

This method may of course be modified such that only cells that have been positively selected are collected and used to extract molecules for analysis. This would require visualization and selection as a prerequisite to gene expression analysis. In the case of an FFPE sample, cells may be obtained followed by RNA extraction, amplification and detection as described herein.

In a further alternative to all of the above, the sequence(s) identified herein may be used as part of a simple PCR or array based assay simply to determine the response to treatment with TAM or another “antiestrogen” agent against breast cancer by use of a sample from a non-invasive or minimally invasive sampling procedure. The detection of sequence expression from samples may be by use of a single microarray able to assay expression of the disclosed sequences as well as other

sequences, including sequences known not to vary in expression levels between normal and non-normal breast cells, for convenience and improved accuracy.

Other uses of the present invention include providing the ability to identify breast cancer cell samples as having different responses to treatment with TAM or another “antiestrogen” agent against breast cancer for further research or study. This provides an advance based on objective genetic/molecular criteria.

The genes identified herein also may be used to generate a model capable of predicting the breast cancer survival and recurrence outcomes of an ER+ breast cell sample based on the expression of the identified genes in the sample. Such a model may be generated by any of the algorithms described herein or otherwise known in the art as well as those recognized as equivalent in the art using gene(s) (and subsets thereof) disclosed herein for the identification of breast cancer outcomes. The model provides a means for comparing expression profiles of gene(s) of the subset from the sample against the profiles of reference data used to build the model. The model can compare the sample profile against each of the reference profiles or against a model defining delineations made based upon the reference profiles. Additionally, relative values from the sample profile may be used in comparison with the model or reference profiles.

In a preferred embodiment of the invention, breast cell samples identified as normal and cancerous from the same subject may be analyzed, optionally by use of a single microarray, for their expression profiles of the genes used to generate the model. This provides an advantageous means of identifying survival and recurrence outcomes based on relative differences from the expression profile of the normal sample. These differences can then be used in comparison to differences between normal and individual cancerous reference data which was also used to generate the model.

Articles of Manufacture

The materials and methods of the present invention are ideally suited for preparation of kits produced in accordance with well known procedures. The invention thus provides kits comprising agents (like the polynucleotides and/or antibodies described herein as non-limiting examples) for the detection of expression of the disclosed sequences. Such kits, optionally comprising the agent

with an identifying description or label or instructions relating to their use in the methods of the present invention, are provided. Such a kit may comprise containers, each with one or more of the various reagents (typically in concentrated form) utilized in the methods, including, for example, pre-fabricated microarrays, buffers, the appropriate nucleotide triphosphates (e.g., dATP, dCTP, dGTP and dTTP; or rATP, rCTP, rGTP and UTP), reverse transcriptase, DNA polymerase, RNA polymerase, and one or more primer complexes of the present invention (e.g., appropriate length poly(T) or random primers linked to a promoter reactive with the RNA polymerase). A set of instructions will also typically be included.

The methods provided by the present invention may also be automated in whole or in part. All aspects of the present invention may also be practiced such that they consist essentially of a subset of the disclosed genes to the exclusion of material irrelevant to the identification of breast cancer survival outcomes via a cell containing sample.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

Examples

Example 1

General methods

Patient and tumor selection criteria and study design

Patient inclusion criteria for this study were: Women diagnosed at the Massachusetts General Hospital (MGH) between 1987 and 2000 with ER positive breast cancer, treatment with standard breast surgery (modified radical mastectomy or lumpectomy) and radiation followed by five years of systemic adjuvant tamoxifen; no patient received chemotherapy prior to recurrence. Clinical and follow-up data were derived from the MGH tumor registry. There were no missing registry data and all available medical records were reviewed as a second tier of data confirmation.

All tumor specimens collected at the time of initial diagnosis were obtained from frozen and formalin fixed paraffin-embedded (FFPE) tissue repositories at the Massachusetts General Hospital.

Tumor samples with greater than 20% tumor cells were selected with a median of greater than 75% for all samples. Each sample was evaluated for the following features: tumor type (ductal vs. lobular), tumor size, and Nottingham combined histological grade. Estrogen and progesterone receptor expression were determined by biochemical hormone binding analysis and/or by immunohistochemical staining as described (Long, A.A. et al. "High-specificity in-situ hybridization. Methods and application." Diagn Mol Pathol 1, 45-57 (1992)); receptor positivity was defined as greater than 3 fmol/mg tumor tissue (Long et al.) and greater than 1% nuclear staining for the biochemical and immunohistochemical assays, respectively.

Study design is as follows: A training set of 60 frozen breast cancer specimens was selected to identify gene expression signatures predictive of outcome or response, in the setting of adjuvant tamoxifen therapy. Tumors from responders were matched to the non-responders with respect to TNM staging and tumor grade. Differential gene expression identified in the training set was validated in an independent group of 20 invasive breast tumors with formalin fixed paraffin-embedded (FFPE) tissue samples.

LCM, RNA isolation and amplification

With each frozen tumor sample within the 60-case cohort, RNA was isolated from both a whole tissue section of 8µm in thickness and a highly enriched population of 4,000-5,000 malignant epithelial cells acquired by laser capture microdissection using a PixCell Iie LCM system (Arcturus, Mountain View, CA). From each tumor sample within the 20-case test set, RNA was isolated from four 8µm-thick FFPE tissue sections. Isolated RNA was subjected to one round of T7 polymerase *in vitro* transcription using the RiboAmp™ kit (frozen samples) or another system for FFPE samples according to manufacturer's instructions (Arcturus Bioscience, Inc., Mountain View, CA for RiboAmp™). Labeled cRNA was generated by a second round of T7-based RNA *in vitro* transcription in the presence of 5-[3-Aminoallyl]uridine 5'-triphosphate (Sigma-Aldrich, St. Louis, MO). Universal Human Reference RNA (Stratagene, San Diego, CA) was amplified in the same manner. The purified aRNA was later conjugated to Cy5 (experimental samples) or Cy3 (reference sample) dye (Amersham Biosciences).

Microarray analysis

A custom designed 22,000-gene oligonucleotide (60mer) microarray was fabricated using ink-jet in-situ synthesis technology (Agilent Technologies, Palo Alto, CA). Cy5-labeled sample RNA and Cy3-labeled reference RNA were co-hybridized at 65°C, 1X hybridization buffer (Agilent Technologies). Slides were washed at 37°C with 0.1X SSC/0.005% Triton X-102. Image analysis was performed using Agilent's image analysis software. Raw Cy5/Cy3 ratios were normalized using intensity-dependent non-linear regression.

A data matrix consisting of normalized Cy5/Cy3 ratios from all samples were median centered for each gene. The variance of each gene over all samples was calculated and the top 25% high variance genes (5,475) selected for further analysis. Identification and permutation testing for significance of differential gene expression were performed using BRB ArrayTools, developed by Dr. Richard Simon and Amy Peng (see [http site at linus.nci.nih.gov/BRB-ArrayTools.html](http://linus.nci.nih.gov/BRB-ArrayTools.html)). Hierarchical cluster analysis was performed with GeneMaths software (Applied-Maths, Belgium) using cosine correlation and complete linkage. All other statistical procedures (two-sample t-test, receiver operating characteristic analysis, multivariate logistic regression and survival analysis) were performed in the open source R statistical environment (see [http site at www.r-project.org](http://www.r-project.org)). Statistical test of significance of ROC curves was by the method of DeLong ("Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach." Biometrics 44, 837-45 (1988)). Disease free survival was calculated from the date of diagnosis. Events were scored as the first distant metastasis, and patients remaining disease-free at the last follow-up were censored. Survival curves were calculated by the Kaplan-Meier estimates and compared by log-rank tests.

Real-Time Quantitative PCR analysis

Real-time PCR was performed on 59 of the 60-case training samples (one case was excluded due to insufficient materials) and the 20-case validation samples. Briefly, 2 µg of amplified RNA was converted into double stranded cDNA. For each case 12ng of cDNA in triplicates was used for real-time PCR with an ABI 7900HT (Applied Biosystems) as described (Gelmini, S. et al. "Quantitative polymerase chain reaction-based homogeneous assay with fluorogenic probes to

measure c-erbB-2 oncogene amplification.” Clin Chem 43, 752-8 (1997)). The sequences of the PCR primer pairs and fluorogenic MGB probe (5' to 3'), respectively, that were used for each gene are as follows:

HoxB13

TTCATCCTGACAGTGGCAATAATC,
CTAGATAGAAAATATGAGGCTAACGATCAT,
VIC- CGATAACCAGTACTAGCTG;

IL17BR

GCATTAACCTAACGATTGGAACTACATT,
GGAAGATGCTTTATTGTTGCATTATC,
VIC-ACAACTTCAAAGCTGTTTTA.

Relative expression levels of HOXB13 in normal, DCIS and IDC samples were calculated as follows. First, all CT values are adjusted by subtracting the highest CT (40) among all samples, then relative expression = $1 / 2^{CT}$.

In Situ Hybridization

Dig-labeled RNA probes were prepared using DIG RNA labeling kit (SP6/T7) from Roche Applied Science, following the protocol provided with the kit. *In situ* hybridization was performed on frozen tissue sections as described (Long et al.).

Table 1. Patients and tumor characteristics of training set.

Sample ID	Tumor type	Size	Grade	Nodes	ER	PR	Age	DFS	Status
1389	D	1.7	2	0/1	Pos	Pos	80	94	0
648	D	1.1	2	0/15	Pos	ND	62	160	0
289	D	3	2	0/15	Pos	ND	75	63	1
749	D	1.8	2	2/9	Pos	Pos	61	137	0
420	D/L	2	3	ND	Pos	Pos	72	58	1
633	D	2.7	3	0/11	Pos	ND	61	20	1
662	D	1	3	6/11	Pos	Pos	79	27	1
849	D	2	1	0/26	Pos	Neg	75	23	1
356	D	1	2	2/20	Pos	ND	58	24	1
1304	D	2	3	0/14	Pos	Pos	57	20	1
1419	D	2.5	2	1/8	Pos	Pos	59	86	0

1093	D	1	3	1/14	Pos	Pos	66	85	0
1047	D/L	2.6	2	0/18	Pos	Neg	70	128	0
1037	D/L	1.5	2	0/4	Pos	Pos	85	83	0
319	D	4	2	1/13	Pos	ND	67	44	1
25	D	3.5	2	0/9	Neg	Pos	62	75	1
180	D	1.6	2	2/19	Pos	Pos	69	169	0
687	D	3.5	3	3/16	Pos	ND	73	142	0
856	D	1.6	2	0/16	Pos	Pos	73	88	0
1045	D	2.5	3	1/12	Pos	Neg	73	121	0
1205	D	2.7	2	1/19	Pos	Pos	71	88	0
1437	D	1.7	2	2/22	Pos	Pos	67	89	0
1507	D	3.7	3	0/40	Pos	Pos	70	70	0
469	D	1	1	0/19	Pos	ND	66	161	0
829	D	1.2	2	0/9	Pos	ND	69	136	0
868	D	3	3	0/13	Pos	Pos	65	130	0
1206	D	4.1	3	0/15	Pos	Neg	84	56	1
843	D	3.4	2	11/20	Pos	Neg	76	122	1
342	D	3	2	9/21	Pos	ND	62	102	1
1218	D	4.5	1	3/16	Pos	Pos	62	10	1
547	D/L	1.5	2	ND	Pos	ND	74	129	1
1125	D	2.6	2	0/18	Pos	Pos	54	123	0
1368	D	2.6	2	ND	Pos	Pos	82	63	0
605	D	2.2	2	6/18	Pos	ND	70	110	0
59	L	3	2	33/38	Pos	ND	70	21	1
68	D	3	2	0/17	Pos	ND	53	38	1
317	D	1.2	3	1/10	Pos	Pos	71	5	1
374	D	1	3	0/15	Pos	Neg	57	47	1
823	D	2	2	0/6	Pos	Pos	51	69	1
280	D	2.2	3	0/12	Pos	ND	66	44	1
651	D	4.7	3	10/13	Pos	ND	48	137	1
763	D	1.8	2	0/14	Pos	Pos	63	118	0
1085	D	4.7	2	0/8	Pos	Pos	48	101	1
1363	D	2.1	2	0/15	Pos	Pos	56	114	0
295	D	3.5	2	3/21	Pos	Pos	52	118	1
871	D	4	3	0/16	Pos	Neg	61	6	1
1343	D	2.5	3	ND	Pos	Pos	79	21	1
140	L	>2.0	2	18/28	Pos	ND	63	43	1
260	D/L	0.9	2	1/13	Pos	ND	73	42	1
297	D	0.8	2	1/16	Pos	Pos	66	169	0
1260	D	3.5	2	0/14	Pos	Pos	58	79	0
1405	D	1	3	ND	Pos	Pos	81	95	0
518	L	5.5	2	3/20	Pos	ND	68	156	0
607	D	1.2	2	5/14	Pos	Pos	76	114	0
638	D	2	2	1/24	Pos	Pos	67	148	0
655	D	2	3	ND	Pos	Pos	73	143	0

772	D	2.5	2	0/18	Pos	Pos	68	69	1
878	D/L	1.6	2	0/9	Pos	Neg	76	138	0
1279	D	2	2	0/12	Pos	Pos	68	102	0
1370	D	2	2	ND	Pos	Pos	73	61	0

Abbreviations: D, ductal; L, lobular; D/L, ductal and lobular features; pos, positive; neg, negative; ND, not determined; ER, estrogen receptor; PR, progesterone receptor; DFS, disease-free survival (number of months); status=1, recurred; status=0, disease-free.

5

Example 2

Identification of differentially expressed genes

Gene expression profiling was performed using a 22,000-gene oligonucleotide microarray as described above. In the initial analysis, isolated RNA from frozen tumor-tissue sections taken from the archived primary biopsies were used. The resulting expression dataset was first filtered based on overall variance of each gene with the top 5,475 high-variance genes (75th percentile) selected for further analysis. Using this reduced dataset, t-test was performed on each gene comparing the tamoxifen responders and non-responders, leading to identification of 19 differentially expressed genes at the P value cutoff of 0.001 (Table 2). The probability of selecting this many or more differentially expressed genes by chance was estimated to be 0.04 by randomly permuting the patient class with respect to treatment outcome and repeating the t-test procedure 2,000 times. This analysis thus demonstrated the existence of statistically significant differences in gene expression between the primary breast cancers of tamoxifen responders and non-responders.

Table 2. 19-gene signature identified by t-test in the Sections dataset

	Parametric p-value	Mean in responders	Mean in non-responders	Fold difference of means	GB acc	Description
1	1.96E-05	0.759	1.317	0.576	<u>AW006861</u>	SCYA4 small inducible cytokine A4
2	2.43E-05	1.31	0.704	1.861	<u>AI240933</u>	ESTs
3	8.08E-05	0.768	1.424	0.539	<u>X59770</u>	IL1R2 interleukin 1 receptor, type II
4	9.57E-05	0.883	1.425	0.62	<u>AB000520</u>	APS adaptor protein with pleckstrin homology and src homology 2 domains
5	9.91E-05	1.704	0.659	2.586	<u>AF208111</u>	IL17BR interleukin 17B receptor
6	0.0001833	0.831	1.33	0.625	<u>AI820604</u>	ESTs
7	0.0001935	0.853	1.459	0.585	<u>AI087057</u>	DOK2 docking protein 2, 56kD
8	0.0001959	1.29	0.641	2.012	<u>AJ272267</u>	CHDH choline dehydrogenase

9	0.0002218	1.801	0.943	1.91	N30081	ESTs, Weakly similar to I38022 hypothetical protein [H.sapiens]
10	0.0004234	1.055	2.443	0.432	AI700363	ESTs
11	0.0004357	0.451	1.57	0.287	AL117406	ABCC11 ATP-binding cassette, sub-family C (CFTR/MRP), member 11
12	0.0004372	1.12	3.702	0.303	BC007092	HOXB13 homeo box B13
13	0.0005436	0.754	1.613	0.467	M92432	GUCY2D guanylate cyclase 2D, membrane (retina-specific)
14	0.0005859	1.315	0.578	2.275	AL050227	Homo sapiens mRNA; cDNA DKFZp586M0723 (from clone DKFZp586M0723)
15	0.000635	1.382	0.576	2.399	AW613732	Homo sapiens cDNA FLJ31137 fis, clone IMR322001049
16	0.0008714	0.794	1.252	0.634	BC007783	SCYA3 small inducible cytokine A3
17	0.0008912	2.572	1.033	2.49	X81896	C11orf25 chromosome 11 open reading frame 25
18	0.0009108	0.939	1.913	0.491	BC004960	MGC10955 hypothetical protein MGC10955
19	0.0009924	1.145	0.719	1.592	AK027250	Homo sapiens cDNA: FLJ23597 fis, clone LNG15281

To refine our analysis to the tumor cells and circumvent potential variability attributable to stromal cell contamination, the same cohort was reanalyzed following laser-capture microdissection (LCM) of tumor cells within each tissue section. Using variance based gene filtering and t-test screening identical to that utilized for the whole tissue section dataset, 9 differentially expressed gene sequences were identified with $P < 0.001$ (Table 3).

Table 3. 9-gene signature identified by t-test in the LCM dataset

	Parametric p-value	Mean in responders	Mean in non-responders	Fold difference of means	GB acc	Description
1	2.67E-05	1.101	4.891	0.225	BC007092	HOXB13 homeo box B13
2	0.0003393	1.045	2.607	0.401	AI700363	ESTs
3	0.0003736	0.64	1.414	0.453	NM_014298	QPRT quinolate phosphoribosyltransferase (nicotinate-nucleotide pyrophosphorylase (carboxylating))
4	0.0003777	1.642	0.694	2.366	AF208111	IL17BR interleukin 17B receptor
5	0.0003895	0.631	1.651	0.382	AF033199	ZNF204 zinc finger protein 204
6	0.0004524	1.97	0.576	3.42	AI688494	FLJ13189 hypothetical protein FLJ13189
7	0.0005329	1.178	0.694	1.697	AI240933	ESTs
8	0.0007403	0.99	1.671	0.592	AL157459	Homo sapiens mRNA; cDNA DKFZp434B0425 (from clone DKFZp434B0425)

9	0.0007739	0.723	1.228	0.589	BC002480	FLJ13352 hypothetical protein FLJ13352
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Only 3 genes were identified as differentially expressed in both the LCM and whole tissue section analyses: the homeobox gene HOXB13 (identified twice as AI700363 and BC007092), the interleukin 17B receptor IL17BR (AF208111), and the voltage-gated calcium channel CACNA1D (AI240933). HOXB13 was differentially overexpressed in tamoxifen nonresponsive cases, whereas IL17BR and CACNA1D were overexpressed in tamoxifen responsive cases. Based on their identification as tumor-derived markers significantly associated with clinical outcome in two independent analyses, the utility of each of these genes was evaluated by itself and in combination with the others.

To define the sensitivity and specificity of HOXB13, IL17BR and CACNA1D expression as markers of clinical outcome, Receiver Operating Characteristic (ROC) analysis (Pepe, M.S. "An interpretation for the ROC curve and inference using GLM procedures." *Biometrics* 56, 352-9 (2000)) was used. For data derived from whole tissue sections, the Area Under the Curve (AUC) values for IL17BR, HOXB13 and CACNA1D were 0.79, 0.67 and 0.81 for IL17BR, HOXB13 and CACNA1D, respectively (see Table 4 and Fig. 1, upper portion). ROC analysis of the data generated from the microdissected tumor cells yielded AUC values of 0.76, 0.8, and 0.76 for these genes (see Table 4 and Fig. 1, lower portion).

Table 4. ROC analysis of using IL17BR, CACNA1D and HOXB13 expression to predict tamoxifen response

	Tissue Sections		LCM	
	AUC	P value	AUC	P value
IL17BR	0.79	1.58E-06	0.76	2.73E-05
CACNA1D	0.81	3.02E-08	0.76	1.59E-05
HOXB13	0.67	0.012	0.79	9.94E-07
ESR1	0.55	0.277	0.63	0.038
PGR	0.63	0.036	0.63	0.033
ERBB2	0.69	0.004	0.64	0.027
EGFR	0.56	0.200	0.61	0.068

AUC, area under the curve; *P* values are AUC > 0.5.

A statistical test of significance indicated that these AUC values are all significantly greater than 0.5, the expected value from the null model that predicts clinical outcome randomly. Therefore, these three genes have potential utility for predicting clinical outcome of adjuvant tamoxifen therapy. As comparison, markers that are currently useful in evaluating the likelihood of response to tamoxifen were analyzed in comparison. The levels of ER (gene symbol ESR1) and progesterone receptor (PR, gene symbol PGR) are known to be positively correlated with tamoxifen response (see Fernandez, M.D., et al. "Quantitative oestrogen and progesterone receptor values in primary breast cancer and predictability of response to endocrine therapy." Clin Oncol 9, 245-50 (1983); Ferno, M. et al. "Results of two or five years of adjuvant tamoxifen correlated to steroid receptor and S-phase levels." South Sweden Breast Cancer Group, and South-East Sweden Breast Cancer Group. Breast Cancer Res Treat 59, 69-76 (2000); Nardelli, G.B., et al. "Estrogen and progesterone receptors status in the prediction of response of breast cancer to endocrine therapy (preliminary report)." Eur J Gynaecol Oncol 7, 151-8 (1986); and Osborne, C.K., et al. "The value of estrogen and progesterone receptors in the treatment of breast cancer." U 46, 2884-8 (1980)).

In addition, growth factor signaling pathways (EGFR, ERBB2) are thought to negatively regulate estrogen-dependent signaling, and hence contribute to loss of responsiveness to tamoxifen (see Dowsett, M. "Overexpression of HER-2 as a resistance mechanism to hormonal therapy for breast cancer." Endocr Relat Cancer 8, 191-5 (2001)). ROC analysis of these genes confirmed their correlation with clinical outcome, but with AUC values ranging only from 0.55 to 0.69, reaching statistical significance for PGR and ERBB2 (see Table 4).

The LCM dataset is particularly relevant, since EGFR, ERBB2, ESR1 and PGR are currently measured at the tumor cell level using either immunohistochemistry or fluorescence *in situ* hybridization. As individual markers of clinical outcome, HOXB13, IL17BR and CAC1D all outperformed ESR1, PGR, EGFR and ERBB2 (see Table 4).

Example 3

Identification of the HOXB13:IL17BR Expression Ratio

HOXB13:IL17BR expression ratio was identified as a robust composite predictor of outcome as follows. Since HOXB13 and IL17BR have opposing patterns of expression, the expression ratio of HOXB13 over IL17BR was examined to determine whether it provides a better composite predictor of tamoxifen response. Indeed, both t-test and ROC analyses demonstrated that the two-gene ratio had a stronger correlation with treatment outcome than either gene alone, both in the whole tissue sections and LCM datasets (see Table 5). AUC values for HOXB13:IL17BR reached 0.81 for the tissue sections dataset and 0.84 for the LCM dataset. Pairing HOXB13 with CACNA1D or analysis of all three markers together did not provide additional predictive power.

Table 5. HOXB13:IL17BR ratio is a stronger predictor of treatment outcome

		t-test		ROC	
		t-statistic	P value	AUC	P value
Tissue Section	IL17BR	4.15	1.15E-04	0.79	1.58E-06
	HOXB13	-3.57	1.03E-03	0.67	0.01
	HOXB13:IL17BR	-4.91	1.48E-05	0.81	1.08E-07
LCM	IL17BR	3.70	5.44E-04	0.76	2.73E-05
	HOXB13	-4.39	8.00E-05	0.79	9.94E-07
	HOXB13:IL17BR	-5.42	2.47E-06	0.84	4.40E-11

AUC, area under the curve; P values are AUC > 0.5.

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The HOXB13/IL17BR ratio was compared to well-established prognostic factors for breast cancer, such as patient age, tumor size, grade and lymph node status (see Fitzgibbons, P.L. et al. "Prognostic factors in breast cancer. College of American Pathologists Consensus Statement 1999." Arch Pathol Lab Med 124, 966-78 (2000)). Univariate logistic regression analysis indicated that only tumor size was marginally significant in this cohort (P=0.04); this was not surprising given that the responder group was closely matched to the non-responder group with respect to tumor size, tumor grade and lymph node status during patient selection. Among the known positive (ESR1 and PGR) and negative (ERBB2 and EGFR) predictors of tamoxifen response, ROC analysis of the tissue sections data indicated that only PGR and ERBB2 were significant (see Table 4). Therefore,

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a comparison of logistic regression models containing the HOXB13:IL17BR ratio either by itself or in combination with tumor size, and expression levels of PGR and ERBB2, were made (see Table 6). The HOXB13:IL17BR ratio alone was a highly significant predictor ($P=0.0003$) and had an odds ratio of 10.2 (95%CI 2.9-35.6). In the multivariate model, HOXB13:IL17BR ratio is the only significant variable ($P=0.002$) with an odds ratio of 7.3 (95%CI 2.1-26). Thus, the expression ratio of HOXB13:IL17BR is a strong independent predictor of treatment outcome in the setting of adjuvant tamoxifen therapy.

Table 6. Logistic Regression Analysis
Univariate Model

Predictor	Odds Ratio	95% CI	P Value
<i>HOXB13:IL17BR</i>	10.17	2.9-35.6	0.0003

Multivariate Model

Predictors	Odds Ratio	95% CI	P Value
<i>Tumor size</i>	1.5	0.7-3.5	0.3289
<i>PGR</i>	0.8	0.3-1.8	0.5600
<i>ERBB2</i>	1.7	0.8-3.8	0.1620
<i>HOXB13:IL17BR</i>	7.3	2.1-26.3	0.0022

All predictors are continuous variables. Gene expression values were from microarray measurements. Odds ratio is the inter-quartile odds ratio, based on the difference of a predictor from its lower quartile (0.25) to its upper quartile (0.75); CI, confidence interval.

Example 4

Independent validation of HOXB13:IL17BR expression ratio

The reduction of a complex microarray signature to a two-gene expression ratio allows the use of simpler detection strategies, such as real-time quantitative PCR (RT-QPCR) analysis. The HOXB13:IL17BR expression ratio by RT-QPCR using frozen tissue sections that were available from 59 of the 60 training cases were analyzed (Fig 2, part a). RT-QPCR data were highly concordant with the microarray data of frozen tumor specimens (correlation coefficient $r=0.83$ for HOXB13, 0.93 for IL17BR). In addition, the PCR-derived HOXB13:IL17BR ratios, represented as ΔCT s, where CT is the PCR amplification cycles to reach a predetermined threshold amount (e.g.,

Fig. 2, parts a and b) and ΔCT is the CT difference between HOXB13 and IL17BR, were highly correlated with the microarray-derived data ($r=0.83$) and with treatment outcome (t test $P=0.0001$, Fig. 2, part c). Thus, conventional RT-QPCR analysis for the expression ratio of HOXB13 to IL17BR appears to be equivalent to microarray-based analysis of frozen tumor specimens.

5 To validate the predictive utility of HOXB13:IL17BR expression ratio in an independent patient cohort, 20 additional ER-positive early-stage primary breast tumors from women treated with adjuvant tamoxifen only at MGH between 1991 and 2000, and for which medical records and paraffin-embedded tissues were available, were identified. Of the 20 archival cases, 10 had recurred with a median time to recurrence of 5 years, and 10 had remained disease-free with a
10 median follow up of 9 years (see Table 7 for details).

Table 7. Patient and tumor characteristics of the validation set.

Sample	Tumor Type	Size	Grade	Nodes	ER	PR	Age	DFS	Status
Test 1	D	1.9	3	0/10	Pos	Pos	69	15	1
Test 2	D	1.7	3	0/19	Pos	Pos	61	117	1
Test 3	D	1.7	2	0/26	Pos	ND	65	18	1
Test 4	D	1.2	2	0/19	Pos	Pos	63	69	1
Test 5	D	1.7	2	2/2	Pos	Pos	60	52	1
Test 6	D	1.1	1	0/10	Pos	Pos	54	59	1
Test 7	D	>1.6	2	0/17	Pos	Neg	66	32	1
Test 8	L	2.6	1-2	0/14	Pos	Pos	58	67	1
Test 9	D	1.2	2	ND	Pos	Pos	93	58	1
Test 10	D	4	3	0/20	Pos	Pos	66	27	1
Test11	D	1.1	2	0/19	Pos	Pos	64	97	0
Test 12	D	2.7	2	0/10	Pos	Pos	66	120	0
Test 13	D	0.9	1	0/22	Pos	Pos	66	123	0
Test 14	D	2.1	2	0/16	Pos	Pos	57	83	0
Test 15	D	0.8	1-2	0/8	Pos	Pos	74	80	0
Test 16	D	1	2	0/13	Pos	Pos	74	93	0
Test 17	D	1.6	2	0/29	Pos	Pos	66	121	0
Test 18	L	1.5	1-2	0/8	Pos	Pos	65	25	0
Test 19	D	1.5	3	0/16	Pos	Pos	60	108	0
Test 20*	L	4	1-2	0/19	Pos	Pos	60	108	0

Abbreviations: Same as supplemental Table 1. * Patient received tamoxifen

for 2 years.

RNA was extracted from formalin-fixed paraffin-embedded (FFPE) whole tissue sections, linearly amplified, and used as template for RT-QPCR analysis. Consistent with the results of the training cohort, the HOXB13:IL17BR expression ratio in this independent patient cohort was highly correlated with clinical outcome (t test $P=0.035$) with higher HOXB13 expression (lower ΔCTs) correlating with poor outcome (Fig. 2, part d). To test the predictive accuracy of the HOXB13:IL17BR ratio, the RT-QPCR data from the frozen tissue sections ($n=59$) was used to build a logistic regression model. In this training set, the model predicted treatment outcome with an overall accuracy of 76% ($P=0.000065$, 95% confidence interval 63%-86%). The positive and negative predictive values were 78% and 75%, respectively. Applying this model to the 20 independent patients in the validation cohort, treatment outcome for 15 of the 20 patients was correctly predicted (overall accuracy 75%, $P=0.04$, 95% confidence interval 51%-91%), with positive and negative predictive values of 78% and 73%, respectively.

Kaplan-Meier analysis of the patient groups as predicted by the model resulted in significantly different disease-free survival curves in both the training set and the independent test set (Fig. 2, parts e and f).

Additional References

Ma, X.J. et al. Gene expression profiles of human breast cancer progression. Proc Natl Acad Sci U S A 100, 5974-9 (2003).

Nicholson, R.I. et al. Epidermal growth factor receptor expression in breast cancer: association with response to endocrine therapy. Breast Cancer Res Treat 29, 117-25 (1994).

All references cited herein, including patents, patent applications, and publications, are hereby incorporated by reference in their entireties, whether previously specifically incorporated or not.

5 Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

10 While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth.

Appendix

Sequences identified as those of IL17BR cluster

5 AW675096

CCGGCGATGTCGCTCGTGCTGCTAAGCCTGGCCGCGCTGTGCAGGAGCGCCGTACCCCGA
GAGCCGACCGTTCAATGTGGCTCTGAACTGGGCCATCTCCAGAGTGGATGCTACAACAT
GATCTAATCCCGGGAGACTTGAGGGACCTCCGAGTAGAACCTGTTACAACCTAGTGTGCA
10 ACAGGGGACTATTCAATTTTGATGAATGTAAGCTGGGTACTCCGGGCAGATGCCAGCATC
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TGTGTGAGGTGCAATTACACAGAGGCCTTCCAGACTCAGACCAGACCCTCTGGTGGTAAA
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15 CTGTGGGATCCGAACATCACT

AW673932

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20 ATCAGTGAGGGGTTCTTAGAGTTAGTATTCATTAATTAAACATAGAATATTAGCTAAACA
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ATAAAACAGCTTTGAAGTTGTAAATGTAGTTTCCAATCGTTAGTTAATGCTACATTAGTT
AGCAATATTTGAAAATTTTATTGGTATAAAATGTTTTAATTACTAAGGCTGTTTGTAGGC
25 TGCATAGTAAGCTTCAGGATCATCACACGTTTTTCCCTGTAATTGG

BC000980

ggcccgccga tgtcgctcgt gctgctaagc ctggccgcgc tgtgcaggag cgccgtaccc
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atccgcttgt tgaaggccac caagatttgt gtgacgggca aaagcaactt ccagtcctac
agctgtgtga ggtgcaatta cacagaggcc ttccagactc agaccagacc ctctggtggt
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35 gcccataata ttcctaataa aaatatgaat gaagatggcc ctcccatgtc tgtgaatttc
acctcaccag gctgcctaga ccacataatg aaatataaaa aaaagtgtgt caaggccgga
agcctgtggg atccgaacat cactgcttgt aagaagaatg aggagacagt agaagtgaac
ttcacaacca ctcccctggg aaacagatac atggctctta tccaacacag cactatcatc
gggttttctc aggtgtttga gccacaccag aagaaacaaa cgcgagcttc agtggtgatt
40 ccagtgactg gggatagtga aggtgctacg gtgcagctga ctccatattt tcctacttgt

ggcagcgact gcatccgaca taaaggaaca gttgtgctct gcccacaaac aggcgtccct
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tagggaataa acaagctgaa cccactttta aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa
aa

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35 CTTTCCCCTCTGGATAACAACAAAAGCAAGCCGGGAGGCTGGCTGCCTCTCCTCCTGCTG
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TG

40

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GTTTACCC

15 BI823321

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AA514396

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40 TTCTGCACAGAAAGCAGTGGCATCCTTCATGAGGTGGTACTTGGGGCAGACACTGAGAGC
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BF740045

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AW299271

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5 AA836217

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AI627783

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AI744263

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AI401622

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AI826949

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BE047352

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AI911549

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AI034244

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AI033911

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AI942234

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AI580483

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AI831909

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AI672344

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AA677205

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BF115018

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20 W61238

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W61239

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AI032064

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AW236941

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AW058532

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40 T98360

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T98361

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AI470845

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AI497731

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T96629

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T96740

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H25975

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H25941

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H14692

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AI298577

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H90761

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AI793318

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AA962325

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W04890

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D25960

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AV709899

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BX431018

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AL535617

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5 AL525465

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BX453536

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BX453537

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20 **AV728945**

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AV728939

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AV727345

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10 CTCTTGAAAGGGCGTG

Sequences identified as those of CACNA1D cluster

BM128550

15 CGGCTCCTACCTTTTGCCCGATCCCCTTCCCCATTCCGCCCCCGCCCCAACGCAGTGCAC
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25 BI755471

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BQ549084

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BQ549571

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AI693324

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R25307

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R46658

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H29256

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H29339

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25 BG716371

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AI537488

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AA458692

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AI393327

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AI520947

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AI248998

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AI075844

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AI869807

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AI869800

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AI243110

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AI955764

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N79331

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N62240.1

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AI015031

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10 BI769856

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AA682690

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BQ898429

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AW872382

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AI680744

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AI601252

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AI459166

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BE672659

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N78509

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N73668

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N46744

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N39597

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BF439267

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BF436153

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BF110611

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M76558

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BE769065

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Sequences identified as those of HOXB13 cluster

BF676461

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BC007092

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BG752489

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CB050884

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BF965191

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BU930208

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AA807966

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AI884491

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20 BQ673782

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20 AA535663

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BF855145

15 CTAGAGGATCCCGGAAGCAACTGCAACAGGTTCCCAAAGAACCGGGCCAGTCATAATCAT
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TATGAATAAATTTGGAAGGCGATCCCTTGGCAGGGAAGCTTTCTCTCAGACCCCTTCCA
TTACACCTCTCACCTGGTAACAGCAGGAAGACTGAGGAGAGGGGAACGGGCAGATTCTGT
20 GGTGTTGCAGTGTGCTTCCG

AU126914

GAGCGAATGCAGGCGACTTGCGAGCTGGGAGCGATTTAAAACGCTTTGGATTCCCCCGGC
CTGGGTGGGGAGAGCGAGCTGGGTGCCCCCTAGATTCCCCGCCCCCGCACCTCATGAGCC
25 GACCCTCGGCTCCATGGAGCCCCGGCAATTATGCCACCTTGGATGGAGCCAAGGATATCGA
AGACTTGCTGGGAGCGGGAGGGGGGCGGAATCTGGTGCGCCACTCCCCTCTGACCAGCCA
CCCAGCGGCGCCTACGCTGATGCCTGCTGTCAACTATGCCCCCTTGGATCTGCCAGGCTC
GGCGGAGCCGCCAAAGCAATGCCACCCATGCCCTGGGGTGCCCCAGGGGACGTCCCCAGC
TCCCGTGCCCTTATGGTTACTTTGGAGGCGGGTNCTACTCCTGCCGAGTGTCCCGGAGCTC
30 GCTGAAACCCTGTGCCCANNCANCCACCCTGGCCGCGTN

CB126449

CTCTGCCTCTGTGTTTCATTCTCTGATGTCCTGTACCTGTGCTCAGTGCCCGGTGGGACTC
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35 GGGGGTGGGGGGCCTGCCGGCGCATCTCCACGATT

AW582404

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TGCAGGGAGACGGAACCCCATGTGACAGCCCACTCCACCAGGGTTCCCAAAGAACCTGGC
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5 TGATCGTTAGCCTCATATTTTCTATCTAGAGCTCTGTAGAGCACTTTAGAAACCGCTTTC
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BX641644

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AGCCTATGGCCAGTTACCTGGACGTGTCTGTGGTGACAGTCTGGGTGCTCCTGGAGAAC
CGCGACATGACTCCCTGTTGCCTGTGGACAGTTACCAGTCTTGGGCTCTCGCTNGTGGCT
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20 CATTTG

Sequences from Table 4 not disclosed above

AW006861 (IMAGE Clone ID: :2497262)

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ACCGCGAGGAAGCTTCCTCGCAACTTTGTGGTAGATTACTATGAGACCAGCAGCCTCTGC
TCCCAGCCAGCTGTGGTATTCCAAACCAAAAGCAAGCAAGTCTGTGCTGATCCCAGT
30 GAATCCTGGGTCCAGGAGTACGTGTATGACCTGGAAGTGAAGTGAAGTCTGCTCAGAGACAG
GAAGTCTTCAGGGAAGGTCACCTGAGCCCGGATGCTTCTCCATGAGACACATCTCCTCCA
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35 CATTTGATTCTGTGTGTTTTTATAATAAACTTTAAATAAAATGCAAAAAAAAAAAAAA
AAAA

X59770

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5 GGAGGACTCTGGCACCTACGTCTGCACTACTAGAAATGCTTCTTACTGTGACAAAATGTC
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10 AGATGCTGGCTATTACCGCTGTGTCTGACATTTGCCCATGAAGGCCAGCAATACAACAT
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AB000520

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5 CAACTTCCAGGGCAAGGCCAAGCACCTGCGCCTGTCCCTGAACGGCCACGGCCAGTGCTCA
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15 GACTGCAAAA

AI820604 (IMAGE Clone Id: 1605108)

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25 TTAACCTTCCCTTTGTTCTTCATGTACACGCTGAACTGCAATTCTTCTTCCCAAATAAAA
CATTAAATTTAAAAAA

AI087057 (IMAGE Clone ID:1671188)

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AJ272267

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N30081 (IMAGE Clone ID: 258695)

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15 AI700363 (IMAGE Clone ID: 2327403)

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AL117406

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20

M92432

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AL050227

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AW613732 (IMAGE Clone ID: 2953502)

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30 ATTTATTTTGTAGACTCTCCACAGCATTATGTCAATATGGGATTAATGCCTAACTTTGT
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BC007783 (IMAGE Clone ID: 4308472)

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X81896

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BC004960 (IMAGE Clone ID: 3632495)

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AK027250

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Sequences from Table 5 not disclosed above

5

NM_014298

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AF033199

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AI688494 (IMAGE Clone ID: 2330499)

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AL157459

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BC002480 (IMAGE Clone ID: 3350037)

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